

STUDIES ON THE ALLOSTERIC CONTROL OF  
YEAST PYRUVATE KINASE

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For Hugh Fischer Culloch, who was more of a father to me than  
he will ever know and is most sadly missed.

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### **DECLARATION**

I HEREBY DECLARE THAT THIS THESIS HAS BEEN COMPOSED BY ME,  
THAT IT HAS NOT BEEN ACCEPTED IN ANY PREVIOUS APPLICATION FOR A  
DEGREE AND THAT THE WORK OF WHICH IT IS A RECORD HAS BEEN  
CARRIED OUT BY ME.



## ABSTRACT

Pyruvate kinase [EC. 2.7.1.40.] is ubiquitous and catalyses the second of the ATP forming reactions of glycolysis, namely the interconversion of phosphoenolpyruvate to pyruvate with concomitant phosphotransfer to ADP to form ATP. The enzyme requires one monovalent and two divalent cations for full activity, one of which is enzyme bound and the others being abstracted to the nucleotide. The enzyme is considered to be the control point for the lower part of the glycolytic pathway.

Pyruvate kinases can be allosterically regulated by a number of effectors which bind to the enzyme at sites distinct from the substrate binding site and modify the enzyme activity. It was the purpose of this project to attempt to determine the nature of this form of regulation using site directed mutagenesis to alter residues suspected to be involved in mediating the allosteric effect.

In this project the pyruvate kinase from the yeast *Saccharomyces cerevisiae* is used as the model for an allosterically regulated pyruvate kinase. This yeast has only one gene encoding a pyruvate kinase (*pyk1*) which is subject to allosteric control. This gene had been cloned and sequenced prior to the outset of this project, however the sequence was seen to contain a number of errors which led me to repeat this process.

The approach adopted in this project was to overexpress pyruvate kinase on a multicopy yeast shuttle vector, this facilitated its subsequent purification. It is hoped that this approach will facilitate the production of very large amounts of protein which can be used for x-ray crystallography and other physico-chemical techniques which require large amounts of protein.

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## ABBREVIATIONS

ADP	ADENOSINE DIPHOSPHATE
AMP	ADENOSINE MONOPHOSPHATE
ATP	ADENOSINE TRIPHOSPHATE
C-	CARBOXY
cAMP	CYCLIC ADENOSINE MONOPHOSPHATE
CdRP	1-(2'-CARBOXYPHENYLAMINO)-1-DEOXYRIBULOSE-5-PHOSPHATE
d-	DEOXY
DNA	DEOXYRIBONUCLEIC ACID
dNTP	DEOXYNUCLOSIDE TRIPHOSPHATE
ds	DOUBLE STRANDED
DTT	DITHIOTHREITOL
DW	DISTILLED WATER
EC	ENZYME COMMISSION NUMBER
EDTA	ETHYLENEDIAMINE TETRAACETIC ACID
FMN	FLAVIN MONONUCLEOSIDE
GTP	GUANINE TRIPHOSPHATE
IGP	INDOLE-3-GLYCEROPHOSPHATE
K	KILO
Kb	KILOBASEPAIRS
Km	MICHAELIS CONSTANT
g	GRAMME
L	LITRE
lb/in <sup>2</sup>	POUNDS PER SQUARE INCH
mA	MILLIAMPERES
mg	MILLIGRAMMES
ml	MILLILITRES
Mr	RELATIVE MOLECULAR MASS
μg	MICROGRAMME
μl	MICROLITRE
N-	AMINO
NADH	NICOTINAMIDE ADENINE DINUCLEOTIDE (REDUCED)
NMR	NUCLEAR MAGNETIC RESONANCE
PAGE	POLYACRYLAMIDE GEL ELECTROPHORESIS
PEG	POLYETHYLENE GLYCOL
PEP	PHOSPHOENOLPYRUVATE
PRA	N-(5'PHOSPHORIBOSYL) ANTHRANYLATE
OD	OPTICAL DENSITY
rpm	REVOLUTIONS PER MINUTE
SDS	SODIUM DODECYL SULPHATE
SDW	STERILE DISTILLED WATER
ss	SINGLE STRANDED
TE	10mM TRIS, 1mM EDTA, pH 8.0
Tm	MELTING TEMPERATURE
TRIS	TRIS[HYDROXYMETHYL]AMINOMETHANE
TTP	THYMINE TRIPHOSPHATE
UTP	URIDINE TRIPHOSPHATE
UV	ULTRAVIOLET
V	VOLTS

## AMINO ACIDS

A  
C  
D  
E  
F  
G  
H  
I  
K  
M  
N  
P  
Q  
R  
S  
T  
V  
W  
Y

ALA  
CYC  
ASP  
GLU  
PHE  
GLY  
HIS  
ILE  
LYS  
MET  
ASN  
PRO  
GLN  
ARG  
SER  
THR  
VAL  
TRP  
TYR

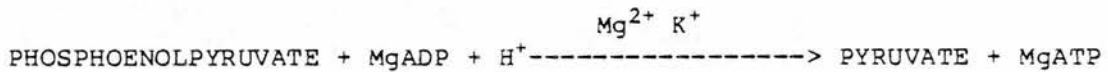
ALANINE  
CYSTEINE  
ASPARTATE  
GLUTAMINE  
PHENYLALANINE  
GLYCINE  
HISTIDINE  
ISOLEUCINE  
LYSINE  
METHIONINE  
ASPARAGINE  
PROLINE  
GLUTAMINE  
ARGININE  
SERINE  
THREONINE  
VALINE  
TRYPTOPHAN  
TYROSINE

## INTRODUCTION

### 1.1. PYRUVATE KINASE

#### 1.1.1. General Introduction

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase) [EC. 2.7.1.40.] catalyses the essentially irreversible reaction;



The enzyme has an absolute requirement for two  $\text{Mg}^{2+}$  and one  $\text{K}^+$  as cofactors, one of the  $\text{Mg}^{2+}$  ions being found in close association with the protein, the other being complexed with the nucleotide. The enzyme has a low nucleotide specificity in that UTP, GTP, CTP, ITP and dADP can all serve as phosphodonors (Strominger, 1955). The properties of the enzyme have been recently reviewed (Muirhead, 1987). The precise reaction mechanism has been studied and will be discussed in considerable detail in section 1.1.8.

Pyruvate kinase catalyses the second of the ATP forming reactions of glycolysis (fig.1) and in this respect is the major control point for the lower part of the pathway. The reaction is essentially irreversible in favour of ATP formation under physiological conditions. Pyruvate is the first non-phosphorylated intermediate in the glycolytic pathway and occupies a central role in metabolism where it may be oxidised to acetyl coenzymeA, its major fate under aerobic conditions, or reduced to form lactate in the absence of oxygen. ATP is used in many anabolic cellular processes, in the transport of molecules across membranes, and in mobility and contractility.

Pyruvate kinases are generally regulated allosterically by effector molecules which bind to the enzyme at a site distinct from the active site. These effectors can act as feed forward activators to signal an increased flux through

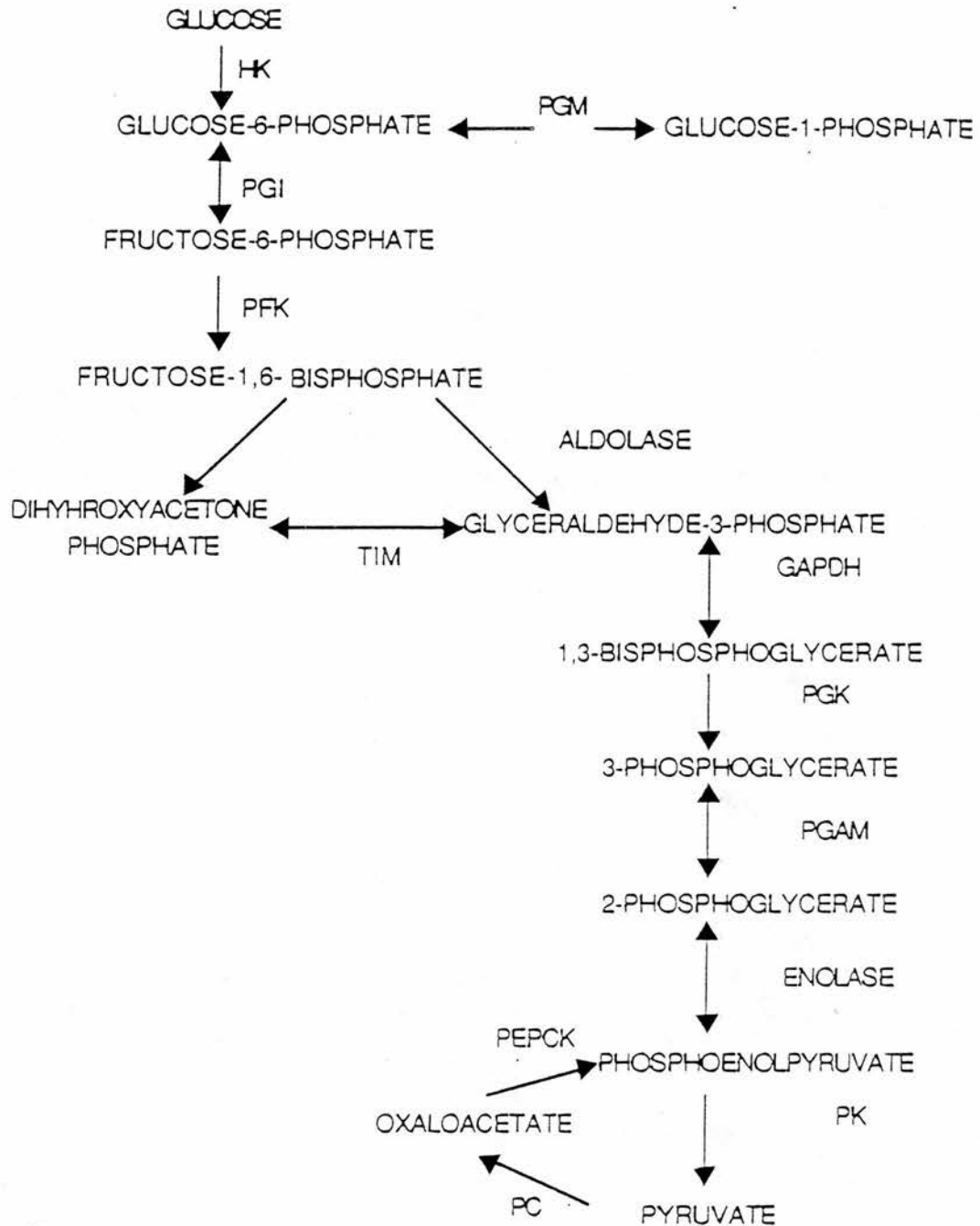


Figure 1; The glycolytic pathway.

THE ABBREVIATIONS FOR THE PATHWAY ARE AS FOLLOWS;  
 HEXOKINASE(HK), PHOSPHOGLUCOMUTASE(PGM), PHOSPHOGLUCO-  
 ISOMERASE(PGI), PHOSPHOFRUCTOKINASE(PFK),TRIOSEPHOSPHATE-  
 ISOMERASE(TIM), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE  
 (GAPDH), PHOSPHOGLYCERATE KINASE(PGK), PHOSPHOGLYCERATE-  
 MUTASE(PGAM), PYRUVATE KINASE(PK), PYRUVATE CARBOXYLASE  
 (PC), PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK)



the pathway. One such activator is fructose-1,6,-bisphosphate (Morris *et al.* 1986). The enzyme may also be feedback inhibited, for example by gluconeogenic amino acids, which signal a high concentration of products of pyruvate metabolism, thus shutting off the enzyme to prevent an unnecessary accumulation of these metabolites. The enzyme is also sensitive to changes in pH and is activated by  $\text{NH}_4^+$  (Kinderler *et al.* 1986, Rhodes *et al.* 1986). Isoenzymes of pyruvate kinase are found in many organisms, both prokaryotic as in *E. coli* and eukaryotic, as in mammalian systems. These isoenzymes are generally classified by their differing regulatory properties and will be discussed in more detail in section 1.1.3.

#### 1.1.2. Sequences of pyruvate kinase

Pyruvate kinase is ubiquitous and has been purified from many sources, both prokaryotic and eukaryotic. This has recently been reviewed and discussed in an evolutionary context (Guderley *et al.* 1989). In general the enzyme is a homotetramer of Mr 260,000 although there are some small species and tissue variations. The only exceptions to this are in the cases of the anaerobic bacterium *Zymomonas mobilis* (Pawluk *et al.* 1988) and the fission yeast *Schizosaccharomyces pombe* (Duncan *et al.* 1989). In both of these cases the molecular weight is approximately half of that of the other pyruvate kinases and it would be tempting to speculate that these may be dimeric forms of the enzyme.

The sequences of 12 pyruvate kinases have now been elucidated, these are illustrated in table 1. In addition some partial peptide sequence has been obtained from *Eschericia coli*, *Bacillus stearothermophilus* and *Zymomonas mobilis* (Speranza *et al.* 1989, H.Muirhead and R. Scopes personal communications) Figure 2 shows a lineup of all known pyruvate kinase

SOURCE	NATURE	SIZE	REFERENCE
CAT M1	PROTEIN	530	Muirhead <i>et al.</i> (1986)
CHICK M1	cDNA	529	Lonberg and Gilbert (1983)
RAT M1	cDNA	530	Noguchi <i>et al.</i> (1985)
RAT M2	cDNA	530	Noguchi <i>et al.</i> (1985)
HUMAN L	cDNA	543	Tani <i>et al.</i> (1988)
RAT L	cDNA	543	Lone <i>et al.</i> (1985)
RAT RBC	cDNA	574	Noguchi <i>et al.</i> (1987)
YEAST	gDNA	500	McNally <i>et al.</i> (1989)
A. nidulans	gDNA	526	de Graaf and Visser (1988)
A. niger	gDNA	526	T. Murcott personal comm.
Trypanosome	gDNA	526	T. Murcott personal comm.

Table 1; The known sequenes of pyruvate kinases from different species and tissues.

\* :=> match across all seqs.  
 . :=> conservative substitutions

```
>catm1      MSKPHS-----DVGTAFTQTQQL
>chickm1    MSKHH-----DAGTAFIQTQQL
>ratm1      -FKPDS-----EAGTAFIQTQQL
>humanliver ME-----GPAGYLRRASVAQLTQELGTAFFQQQQL
>ratliver   ME-----GPAGYLRRASVAQLTQELGTAFFQQQQL
>ratrbc     MSVQENTLPQQLWPWIFRSQKDLAKSALSGAGGPAGYLRRASVAQLTQELGTAFFQQQQL
>ratm2      -PKPDS-----EAGTAFIQTQQL
>yeast      MS-----R-----
>aspidulans MAAS-----SSLDHLSNRMK-----
>aspidiger  MAAS-----SSLDHLSNRMK-----
>trypanosome1 MS-----GLEHNIGLSIFE-----
>trypanosome2 MS-----GLEHNIGLSIFE-----
>bacillus   M-K-----RK TK-----FVS-----
>Zmobilis   -TEGLFPRG-----RKVR-----VVS-----
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```
>catm1      HAAMADTFLEHMCRLDIDSPPIA-RNTGI ICTIGPASRSVEILKEMIKSGMNVARLNFS
>chickm1    HAAMADTFLEHMCRLDIDSEPIA-RNTGI ICTIGPASRSVDKLEKEMIKSGMNVARLNFS
>ratm1      HAAMADTFLEHMCRLDIDSAPIA-RNTGI ICTIGPASRSVEMLKEMIKSGMNVARLNFS
>humanliver PAAMADTFLEHLCCLLDIDSEPVAA-RSTSI IATIGPASRSVGRLEKEMIKAGMNIARLNFS
>ratliver   PAAMADTFLEHLCCLLDIDSEPVAA-RSTSI IATIGPASRSVDRLEKEMIKAGMNIARLNFS
>ratrbc     PAAMADTFLEHLCCLLDIDSEPVAA-RSTSI IATIGPASRSVDRLEKEMIKAGMNIARLNFS
>ratm2      HAAMADTFLEHMCRLDIDSAPIA-RNTGI ICTIGPASRSVEMLKEMIKSGMNVARLNFS
>yeast      -----LERLTSLN--VVAGSDLRRTSI IGTIGPK TNNPETLVALLKAGLNIVRMNFS
>aspidulans -----LEWHSKLNTMVPAKNFRRTSI ICTIGPK TNSVEK INALRAGLNIVRMNFS
>aspidiger  -----LEWHSKLNTMVPAKNFRRTSI IGTIGPK TNSVEK INALRTAGLNIVRMNFS
>trypanosome1 -----FVAKHRANRIVCTIGPSTQSVEALKNLKSGMSVARMNFS
>trypanosome2 -----FVAKHRANRIVCTIGPSTQSVEALKNLKSGMSVARMNFS
>bacillus   -----TFG-----PASESVDKLVQLMEAGMNVARNLFS
>Zmobilis   -----TLG-----PASS-TEAQ-----FL
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. . . \* . \* . \* . \* . . . . . \* . . . . \* . \* . \* .

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>catm1      HGTHEYHAETIKNVRAATESFASDFIRYRFVAVALDTKGPEIRTGLIKSGSGTAEEVELKKG
>chickm1    HGTHEYHEGTIKNVREATESFASDFITYRFVAIALDTKGPEIRTGLIKSGSGTAEEVELKKG
>ratm1      HGTHEYHAETIKNVRAATESFASDFILYRFVAVALDTKGPEIRTGLIKSGSGTAEEVELKKG
>humanliver HGSHEYHAETIANVREAVESFAGSFLSYRFVAIALDTKGPEIRTGILQGGPESEVEIVKG
>ratliver   HGSHEYHAESIANIREATESFATSPLSYRFVAIALDTKGPEIRTGVLQGGPESEVEIVKG
>ratrbc     HGSHEYHAESIANIREATESFATSPLSYRFVAIALDTKGPEIRTGVLQGGPESEVEIVKG
>ratm2      HGTHEYHAETIKNVRAATESFASDFILYRFVAVALDTKGPEIRTGLIKSGSGTAEEVELKKG
>yeast      HGSYEHYKSVIDNARKSEELYFG-----RPLAIALDTKGPEIRTG---TTTNDVDYPIPPN
>aspidulans HGSYEHYQSVIDHAREAEKQAG-----RPVAIALDTKGPEIRTG---NTVGDKDIPKAG
>aspidiger  HGSYEHYQSVIDHAREAAKQVG-----RPLAIALDTKGPEIRTG---NPDGDKDIPKQG
>trypanosome1 HGSHEYHQT TINNVRAAAELGLH-----IGIALDTKGPEIRTGFLK---QGEVSFAPG
>trypanosome2 HGSHEYHQT TINNVRAAAELGLH-----IGIALDTKGPEIRTGFLK---QGEVTFAPG
>bacillus   HGDHERH-----GRR IANT
>Zmobilis   AGD
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\*\* . . . \* . \* . . .

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Figure 2; Sequence alignment of pyruvate kinase sequences generated by the multiple sequence alignment programme CLUSTAL, Higgins and Sharp (1988).

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>catm1      ATLK I TLDNAYMEK CDENVLWLDYKNICKVVEVGSKIYVDDGLISLLVKEKGAD-FLVTE
>chickm1    AALKVTLDNAFMENC DENVLWVDYKNLIKVIDVGSKIYVDDGLISLLVKEKGAD-FVMTE
>ratm1      ATLK I TLDNAYMEK CDENVLWLDYKNICKVVEVGSKIYVDDGLISLVKEKGAD-YLVTE
>humanliver SQVLVTVDFAFRTGRNANTVWVDYFNIVRVVPVGGRIYIDDGLISLVVQKISFE-GLVTD
>ratliver   SQVLVTVDFAFQTRGDAKTVWVDYHNI TRVVAVGGRIYIDDGLISLVVQKIGFE-GLVTE
>ratrbc     SQVLVTVDFAFQTRGDAKTVWVDYHNI TRVVAVGGRIYIDDGLISLVVQKIGFE-GLVTE
>ratm2      ATLK I TLDNAYMEK CDENVLWLDYKNICKVVEVGSKIYVDDGLISLVKEKGAD-YLVTE
>yeast      HEMI FTDDKYAKACDDKIMYVDYKNITKVISAGRIYVDDGLVLSFQVLEVVDOKTLKVK
>aspidulans HEMNISTDEQYATASDDQNMVYDYKNITKVISAGKLIYVDDGILSFEVLEVVDOKTLFVR
>aspniger   HELNITTTDEQYATASDDKNMYLDYKNITKVISAGKLIYVDDGILSFEVLEVVDOKTIRVR
>trypanosome1 DIVCVTTDPAYEKVGTEKFYIDYPLTNAVPVGGSIYVDDGVMTLRVVSKEDORTLCKH
>trypanosome2 DIVCVTTDPAYEKVGTEKFYIDYPLTNAVPVGGSIYVDDGVMTLRVLSKEDORTLCKH

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\* \* \* \* \*

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>catm1      VENGGSLSGSKKGVNLPGAAVDLPAVSEKDIDQLKFGVEQDVDMVFASFIRKASDVHEVRK
>chickm1    VENGGMLSGSKKGVNLPGAAVDLPAVSEKDIDQLKFGVEQDVDMVFASFIRKAADVHAVRK
>ratm1      VENGGSLSGSKKGVNLPGAAVDLPAVSEKDIDQLKFGVEQDVDMVFASFIRKAADVHEVRK
>humanliver VENGGLVLSRKGVNLPGAQVLDPLGLSEQDVLRLRFGVGHVQDIIVFASFVRKASDVAAVRA
>ratliver   VENGGLVLSRKGVNLPNTVDLPLGLSEQDVLRLRFGVGHVQDIIVFASFVRKASDVLAVRD
>ratrbc     VENGGLVLSRKGVNLPNTVDLPLGLSEQDVLRLRFGVGHVQDIIVFASFVRKASDVLAVRD
>ratm2      VENGGSLSGSKKGVNLPGAAVDLPAVSEKDIDQLKFGVEQDVDMVFASFIRKAADVHEVRK
>yeast      ALNAGKICSHKGVNLPSTVDLPLALSEKDKEDLRFVGNVGMVFASFIRTAADVLTIRE
>aspidulans CLNNGNISSRKGVNLPSTVDLPLALSEKDIDQLKFGVKNKVDMLVASFIRRGSDIRHIRE
>aspniger   CLNNGNISSRKGVNLPSTVDLPLALSEKDIDQLKFGVKNKVDMLVASFIRRGSDIRHIRE
>trypanosome1 VNNHRLTDRRGINLPGEVDLPAVSEKDRKDLFGBVAEGVDMIFASFIRTAEQVREVRA
>trypanosome2 VNNHRLTDRRGINLPGEVDLPAVSEKDRKDLFGBVAEGVDMIFASFIRTAEQVREVRA

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>catm1      VLGEKGKNIK IISK IENHEGVRRFDEILEASDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>chickm1    VLGEKGKHIK IISK IENHEGVRRFDEIMEASDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>ratm1      VLGEKGKNIK IISK IENHEGVRRFDEILEASDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>humanliver ALGPEGHGK IISK IENHEGVKRFDEILEVSDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>ratliver   ALGPEGQNIK IISK IENHEGVKKFDEILEVSDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>ratrbc     ALGPEGQNIK IISK IENHEGVKKFDEILEVSDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>ratm2      VLGEKGKNIK IISK IENHEGVRRFDEILEASDGMVARGDLGIEIPAEEKVFLAQKMMIGR
>yeast      VLGEQKGDKV IIVK IENQGGVNNFDEILKVTGVMVARGDLGIEIPAPEVLAVQKKLIAK
>aspidulans VLGEEGREIQ IIAK IENQGGVNNFDEILEETDGVMVARGDLGIEIPAPKVFIAQKMMIAK
>aspniger   VLGEEGREIQ IIAK IENQGGVNNFDEILEETDGVMVARGDLGIEIPAPKVFIAQKMMIAK
>trypanosome1 ALGEKGKDIL IISK IENHQGVNIDSI IEASNGIMVARGDLGVEIPAEEKVCAEMCIISK
>trypanosome2 ALGEKGKDIL IISK IENHQGVNIDSI IEASNGIMVARGDLGVEIPAEEKVCAEMCIISK

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>Ecoli      AEAGDVANA ILDBTDAVMLSGESAKGK
>catm1      CNRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYFLEAVR
>chickm1    CNRAGKPIICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYFLEAVR
>ratm1      CNRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYFLEAVR
>humanliver CNLAGKPVVCATQMLESMITKPRPTRAETSDVANAVLDGADCIMLSGETAKGNFFVEAVK
>ratliver   CNLAGKPVVCATQMLESMITKPRPTRAETSDVANAVLDGADCIMLSGETAKGNFFVEAVM
>ratrbc     CNLAGKPVVCATQMLESMITKPRPTRAETSDVANAVLDGADCIMLSGETAKGNFFVEAVM
>ratm2      CNRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYFLEAVR
>yeast      SNLAGKPVICATQMLESMITNPRPTRAESDVGNAILDGADCVMLSGETAKGNYPINAVT
>aspidulans CNIKGKPVICATQMLESMITNPRPTRAESDVANAVLDGADCVMLSGETAKGNYPCEAVT
>aspniger   CNIKGKPVICATQMLESMITNPRPTRAESDVANAVLDGADCVMLSGETAKGNYPNEAVK
>trypanosome1 CNVVGKPVICATEMLESMTNPRPTRAESDVANAVLNGADCVMLSGETALGKYPNEVVQ
>trypanosome2 CNVVGKPVICATEMLESMTNPRPTRAESDVANAVLNGADCVMLSGETALGKYPNEVVQ

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>Ecoli                                LDAPLIVVATNGGK
>catm1                               MQHLIAREAEAAAMFHRKLFEELVRGSSSHSTDLMEAMAMGSVEASYKCLAAALI-VLTESGR
>chickm1                             MQHAIAAREAEAAAMFHRQWFEEILRHSVHHREPADAMAAGAVEASFYKCLAAALI-VMTESGR
>ratm1                               MQHLIAREAEAAVHRLLFEELARASQSTDPLEAMAMGSVEASYKCLAAALI-VLTESGR
>humanliver                         MQHRIAREAEAAVYHRQLFEELRRAAPLSRDPTEVTAIGAVEAAFKCCAAAI-VLTTTGR
>ratliver                           MQHAIAAREAEAAVYHRQLFEELRRAAPLSRDPTEVTAIGAVEASFYKCCAAAI-VLTTTGR
>ratrbc                             MQHAIAAREAEAAVYHRQLFEELRRAAPLSRDPTEVTAIGAVEASFYKCCAAAI-VLTTTGR
>ratm2                              MQHLIAREAEAAIYHLQLFEELRRLAPITSDPTEAAAVGAVEASFYKCCSGAI-VLTKSGR
>yeast                              TMAETAVIAEQAIAYLPNYDDMRNCTFKPTSTTTETVAASAVAAVFEQKAKAI-VLSTSGT
>aspidulans                         MMSETCLLAEVAIPHFNVFDELRLNAPRFTDTVESIAMAASVSLGLNAGAI-VLTTSGN
>aspniger                           MMSETCLLAEVAIPHFNVFDELRLNAPRFTDTVESIAMAASVSLGLNAGAI-VLTTSGK
>trypanosome1                      YMARICVEAQSAETHDTVMFNSIKNLQKIPMCPEEAVCSSAVASAFEVQAKAML-VLSTTGR
>trypanosome2                      YMARICVEAQSAETHDTVMFNSIKNLQKIPMCPEEAVCSSAVASAFEVQAKAML-VLSTTGR
```

```

>Ecoli SAR K Y F P D A T I L A L T D N E K
>catm1 SAHQVARYRPRAP I I A V T R N H G T A R Q A H L Y R G I F F V V C K D P V D E ---- A W A E D V D L R V N L
>chickm1 SAHLVSRYRPRAP I I A V T R N D Q T A R Q A H L Y R G V F P V L C K Q P A H D ---- A W A E D V D L R V N L
>ratm1 SAHQVARYRPRAP I I A V T R N P Q T A R Q A H L Y R G I F F V L C K D A V L D ---- A W A E D V D L R V N L
>humanliver SAQLLSRYRPRAAV I A V T R S A Q A A R Q V H L C R G V F P L L Y R E P P E A ---- I W A D D V D R R V Q F
>ratliver SAQLLSQYRPRAAV I A V T G S A K A A R Q V H L S R G V F P L L Y R E P P E A ---- I W A D D V D R R V Q F
>ratrbcb SAQLLSQYRPRAAV I A V T G S A K A A R Q V H L S R G V F P L L Y R E P P E A ---- I W A D D V D R R V Q F
>ratm2 SAHQVARYRPRAP I I A V T R N P Q T A R Q A H L Y R G I F P V L C K D A V L D ---- A W A E D V D L R V N L
>yeast T P R L V S K Y R P N C P I I L V T R C P R A A R F S H L Y R G V F P F V F - E K E P ---- V S D W T D D V E A R I N F
>aspidulans T A R M I S K Y R P V C P I I M V S R N P A A T R Y S H L Y R G V W P F Y F P E K K P D F N V K I W Q E D V D R R L K W
>aspniger T A R Y L S K Y R P V C P I I M V T R N P A A S R Y S H L Y R G V W P F Y F P E K K P D F N V K V W Q E D V D R R L K W
>trypanosome1 S A R L I S K Y R P N C P I I C V T T R L Q T C R Q L N V T R S V S V F Y D A A K S G E D ----- K D K E K R V K L
>trypanosome2 S A R L I S K Y R P N C P I I C V T T R L Q T C R Q L N V T R S V S V F Y D A A K S G E D ----- K D K E K R V K L

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>catm1      AMNVGKARGFFFKHGDVVIVLTGWRPSSGFTNTMRVVPVP-----
>chickm1    GMNVGKARGFFK TGD L V I V L T G W R P S S G Y T N T M R V V P V P -----
>ratm1      AMNVGKARGFFFKGDVVIVLTGWRPSSGFTNTMRVVPVP-----
>humanliver GIESGKLRGFLRVGDLIVVVTGWRPSSGYTNIMRVLSIS-----
>ratliver   GIESGKLRGFLRVGDLIVVVTGWRPSSGYTNIMRVLSVS-----
>ratrbc     GIESGKLRGFLRVGDLIVVVTGWRPSSGYTNIMRVLSVS-----
>ratm2      AMNVGKARGFFFKGDVVIVLTGWRPSSGFTNTMRVVPVP-----
>yeast      GIEAKAEFGILKKGDITYVSIQGFKAGAGHSNTLQVSTV-----
>aspidulans GINHGLKLG I I N K G D N I V C V Q G W R G G M G H T N T V R V V P A E E N L G L S E
>aspniger   GINHALKLG I I N K G D N I V C V Q G W R G G M G H T N T V R V V P A E E N L G L A E
>trypanosome1 GLDFAKKEKYASTGDVVVVVVHADHSVKGYFPNQTRLIYLP-----
>trypanosome2 GLDFAKKEKYASTGDVVVVVVHADHSVKGYFPNQTRLIYLP-----

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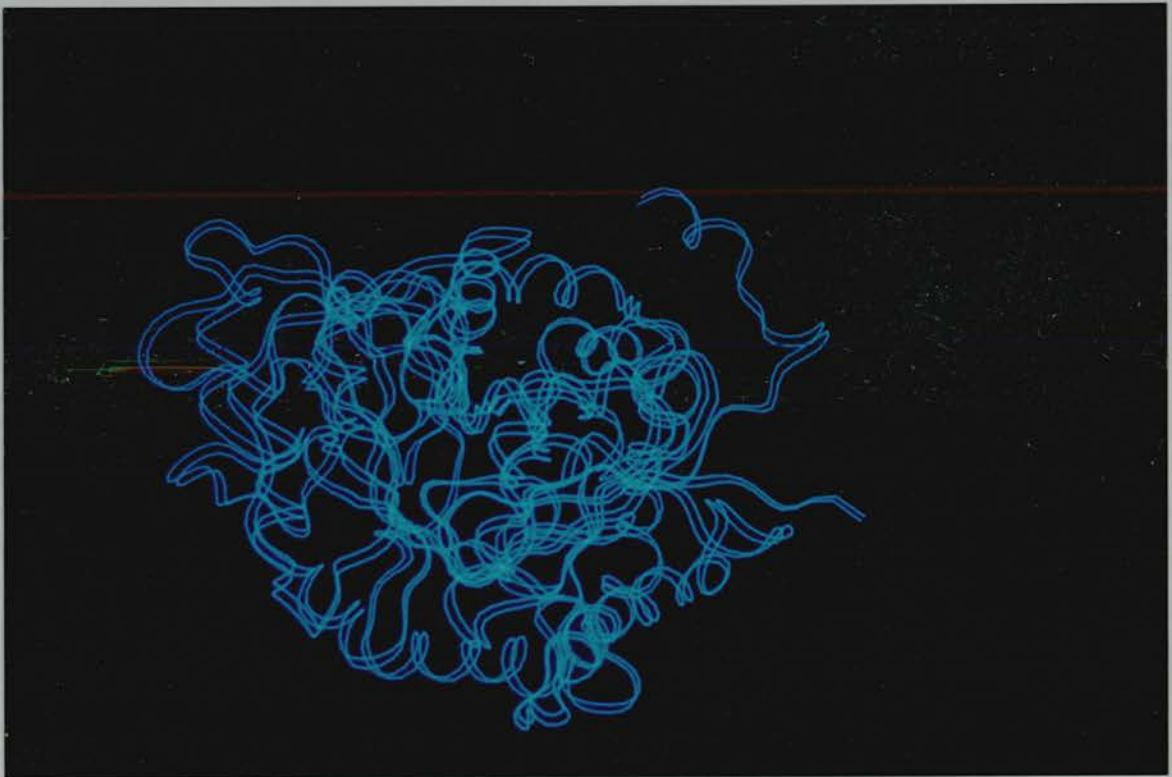
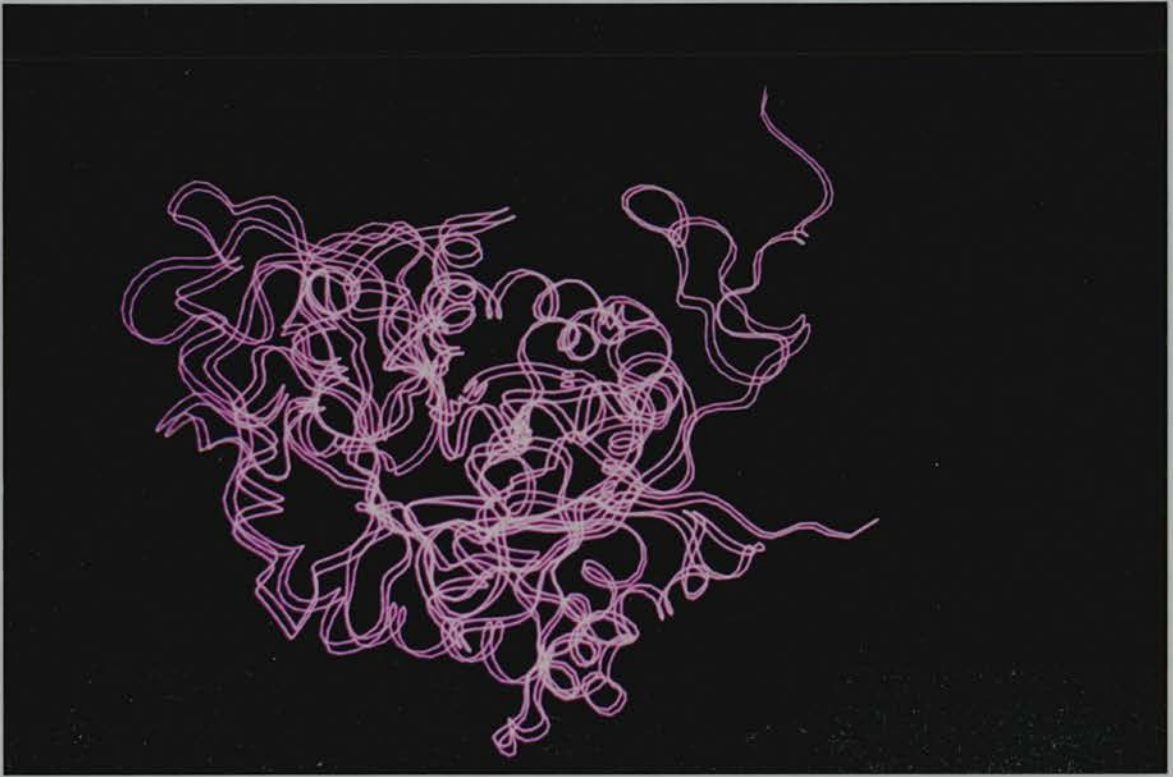


Figure 3; (a) Monomer of cat muscle pyruvate kinase (b) Monomer of yeast pyruvate kinase.

sequences which was generated using the multiple sequence alignment program CLUSTAL (Higgins and Sharp, 1988) Partial protein sequences from *E.coli* and *B.stearothermophilus* have been added later and aligned to the other sequences by eye. The N terminal sequence of *Z.mobilis* is inserted but has no sequence similarity to any of the other pyruvate kinases. In mammalian systems four different isoenzymes of pyruvate kinase have been demonstrated and these are illustrated in table 2. Sequence information is available for examples of each of these different isoenzymes from a number of organisms.

### 1.1.3. Regulation of pyruvate kinase

Pyruvate kinases are subject to both coarse and fine control. The mammalian liver isoenzyme is subject to coarse control by phosphorylation of a specific serine residue under hormonal control by cAMP dependant protein kinase. The specific phosphoserine residue has been identified and is located near the N-terminus of the protein. Phosphorylation at this serine residue leads to a decrease in the enzyme affinity for PEP and fructose 1,6 bisphosphate, and an increase in the affinity for ATP and alanine with the  $V_{max}$  remaining unaffected (Bergstrom *et al.*, 1976), Eckman *et al.*, 1976). These changes are reversed by dephosphorylation of the enzyme and serve to prevent a futile loop between glycolysis and gluconeogenesis.

Yeast pyruvate kinase is also reported to be phosphorylated by cAMP-dependant protein kinase *in vitro* (Blair and Harman, 1986). Structural studies reveal that this cannot be at the same position as in liver pyruvate kinase as the yeast enzyme has a truncated amino terminus and lacks the corresponding phosphorylated serine residue. Sequencing studies reveal the presence of a potential cAMP-dependant protein kinase binding site in yeast pyruvate kinase (Burke *et al.*, 1983). Phosphorylation appears to activate the

PROPERTY	M 1	M 2	L	RBC
TISSUE DISTRIBUTION	MUSCLE	KIDNEY	LIVER	ERYTHROID CELLS
ALLOSTERIC CONTROL	-	+	+	+
INHIBITION BY PHOSPHORYLATION	-	-	+	-

Table 2; The four mammalian isoenzymes of pyruvate kinase.



enzyme, however the authors report that their results are variable and enzyme preparation dependant, therefore the physiological significance of this phosphorylation event, if any, has not been elucidated. Both the liver and yeast PKs are also subject to fine control by allosteric effectors.

The M1 muscle isoenzyme is reported to be regulated by a reversible binding to F-actin. There are said to be 4 pyruvate kinase binding sites per turn of the F-actin helix and binding is reported to decrease enzyme activity by 60% (Chan *et al.*, 1986). The M1 isoenzyme is the only isoenzyme so far reported that is insensitive to all allosteric effectors under physiological conditions.

Eukaryotic pyruvate kinases from certain sources are subject to fine control by allosteric effectors, reflecting the different metabolic requirements of the cells in which they are located. These regulated forms are activated by fructose 1,6 bisphosphate and are inhibited by ATP, gluconeogenic amino acids, citrate and high pH. Prokaryotic pyruvate kinases are also allosterically regulated. The gram negative bacterium *Eschericia coli* has two forms of the enzyme both of which are subject to allosteric control. One form is eukaryotic-like in that it is activated by fructose 1,6 bisphosphate while the other is activated by AMP, ribose-5-phosphate and other nucleoside monophosphates (Speranza *et al.*, 1989.) The gram positive, moderate thermophile, *Bacillus stearothermophilus* has only one form of the enzyme, that which is regulated by AMP (Sakai *et al.*, 1986). This would appear to reflect the different metabolic requirements of prokaryotic cells. While all bacteria so far studied, including archaeobacteria, appear to have a pyruvate kinase, not all of them utilise the Embden-Mayerhoff pathway of glycolysis as the main pathway for the catabolism of glucose. Many lower organisms utilise the Entner-Doudroff pathway and glucose-6-phosphate shunt as mechanisms of

glucose catabolism, and thus the enzymes appear to have evolved to respond to the products of these pathways. The fact that *E.coli* has two pyruvate kinases reflects its ability to undertake both of these metabolic pathways.

The yeast *Saccharomyces cerevisiae* has only one form of pyruvate kinase which is 'eukaryotic' in that it is allosterically regulated by fructose-1,6-bisphosphate, ATP and gluconeogenic amino acids, just as the mammalian isoenzymes are. The enzyme is encoded by the gene *pyk1*, located on chromosome 1. Yeasts, as lower eukaryotes, have only one pyruvate kinase which is allosterically regulated. This may be a reflection of their undifferentiated form.

#### 1.1.4. The structure of pyruvate kinase

An X-ray structure of the M1 isoenzyme of pyruvate kinase from cat muscle has been elucidated and resolved to 2.6Å (Muirhead *et al.*, 1986). Figure 3a illustrates a monomer of pyruvate kinase while figure 4 demonstrates the tetrameric arrangement of the protein. Each monomer is arranged in four domains designated N, A, B, and C as illustrated in figure 5. Domain N runs from residue 1-42, A from residue 43-115 then 224-387, B from 116-223 and C from 388-530. The N terminal residues 1-9 cannot be assigned in the electron density map, probably a reflection of their inherent flexibility. Some of the B domain also appears to be disordered. Domain A forms an eight-stranded  $\alpha/\beta$  barrel, a common element in protein tertiary structure (Richardson, 1981) which will be discussed in section 1.2 in considerably more detail.

The active site of the protein lies in a cleft between domains A and B, while the allosteric effectors bind between domains A and C. Figure 6 demonstrates the positions of the substrates and effectors within the monomer. The tetrameric protein may be considered to be a dimer of dimers in that there are

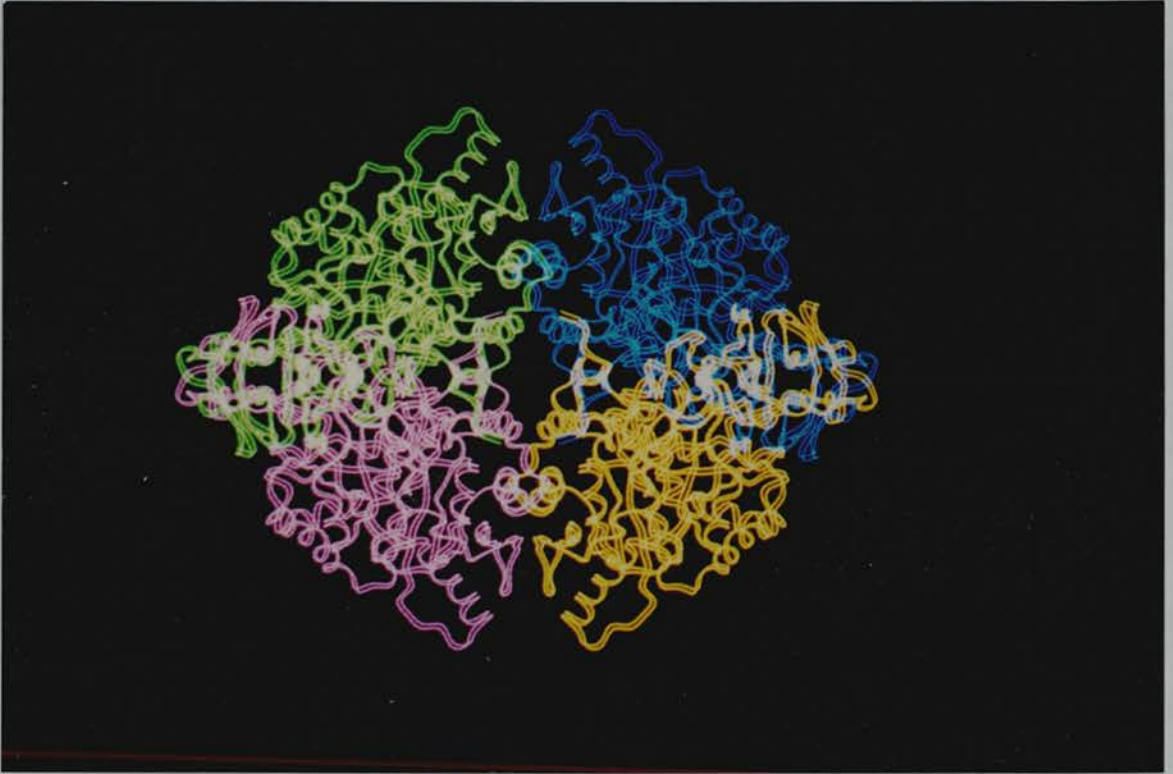


Figure 4; Tetramer of pyruvate kinase.

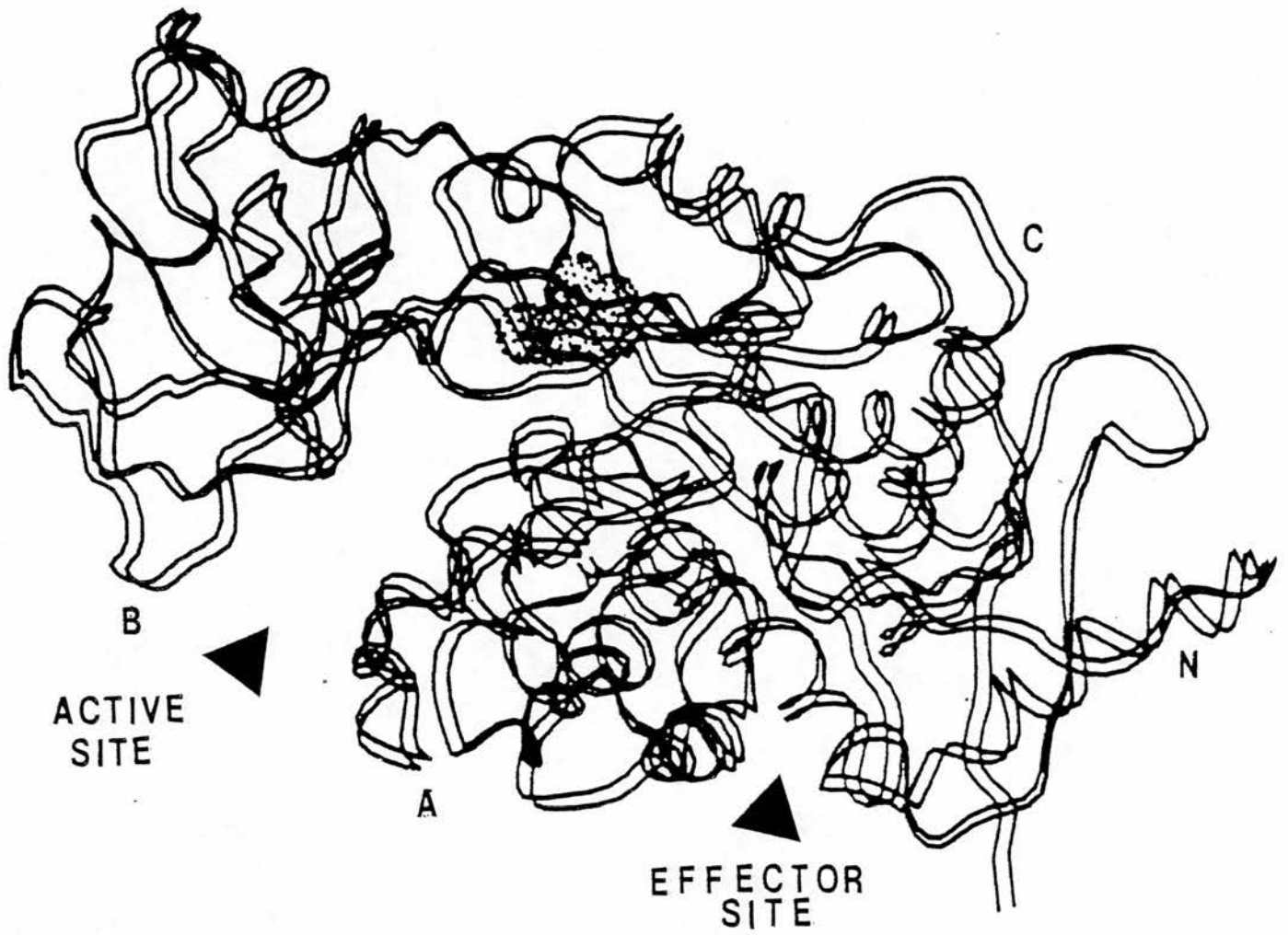


Figure 5; Monomer of cat muscle pyruvate kinase illustrating the 4 domains,

A, B, C and N

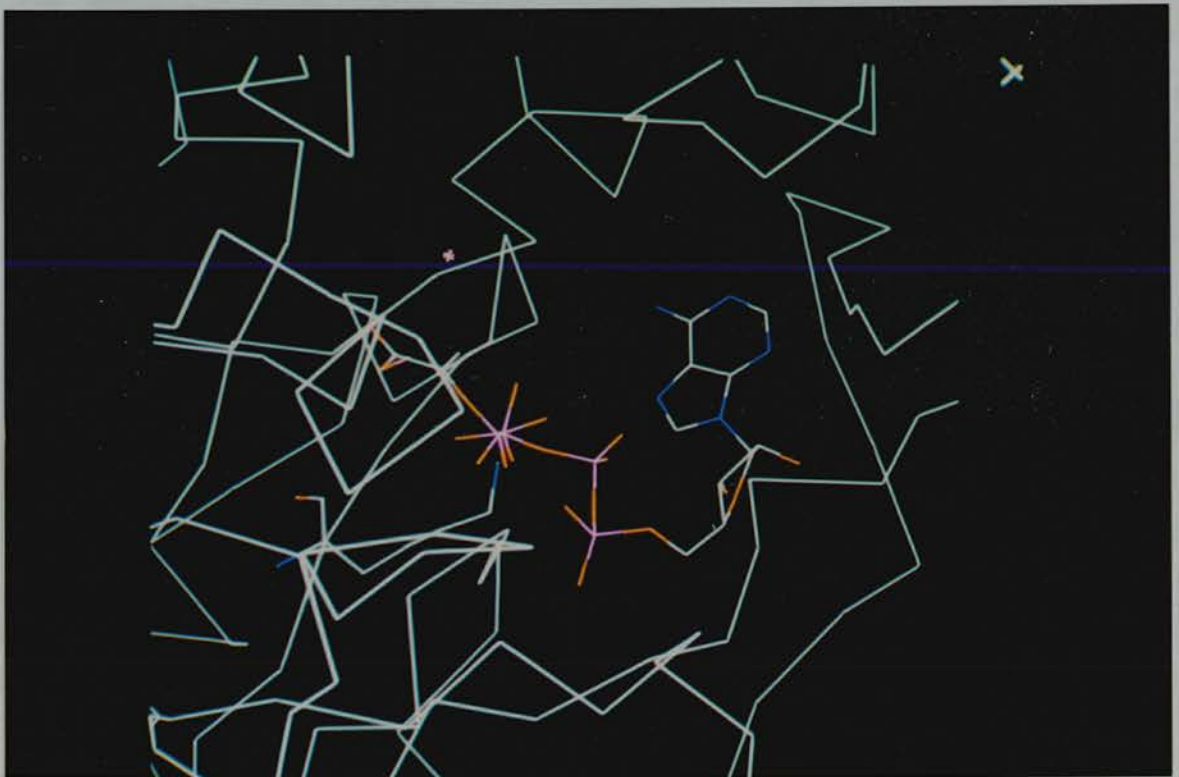
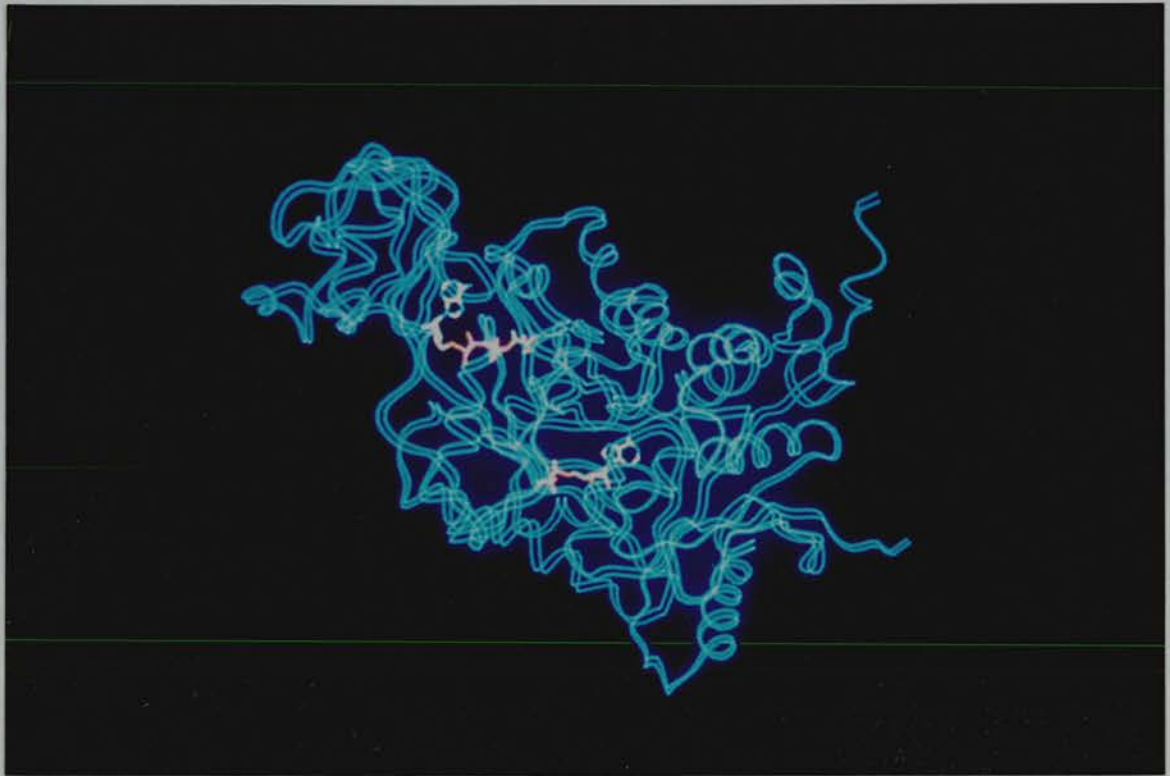


Figure 6; A monomer of pyruvate kinase is illustrated in the upper diagram, with the substrates and allosteric effectors in position. Phosphoenolpyruvate and ATP are shown in the substrate binding site. ATP is bound in the effector binding site. The lower figure shows, in close up, the overlapping phosphates of ATP and PEP. The side chain of lysine 269 is shown in blue. This is implicated as the catalytic lysine.



two different types of intersubunit contact (fig 7). The 1,2 intersubunit contact is the major contact region involving both domains A and C. There is considerably less contact in the 1,3 intersubunit contact region which involves only the C domain and specifically the two  $\alpha$ -helices C $\alpha$ 1 and C $\alpha$ 2. In those enzymes which are reported to be dimeric, it would seem reasonable to speculate that dimerisation occurred across this considerably less extensive interface.

From the point of view of this project which involves an investigation into the allosteric nature of pyruvate kinase, it would be a great advantage if the structure of one of the allosteric forms of the enzyme were available. However, studies of this nature have been hampered by the inability to grow suitable crystals of any of the allosteric forms of the enzyme. Reports have centred on the pyruvate kinase from the yeast *Saccharomyces carlsbergensis*, a close relative of *Saccharomyces cerevisiae*, however the crystals obtained from this source have not been found to be suitable for X-ray analysis (Hess and Sosskina, 1978). The pyruvate kinase from *Saccharomyces cerevisiae* is 65% identical, at the amino acid level, to the cat muscle enzyme, thus they may be assumed to be structurally homologous. A model of the 3D structure of the yeast enzyme was generated by Mr. T.H.L. Murcott, Department of Biochemistry, University of Bristol, by superimposing the the yeast primary structure on to the cat muscle co-ordinates using the Evans and Sutherland graphics facility and the computer package FRODO. Access to this model was generously provided by Dr. H. Muirhead and Mr. Murcott, which proved to be invaluable in these studies. This model represents the structure of the yeast pyruvate kinase in the T-state. The approach adopted in this project, to purify pyruvate kinase from an overexpressing strain of yeast should yield yeast pyruvate kinase in higher quantity than previously available. The enzyme can also be obtained with a higher specific activity than was previously possible. Both of these

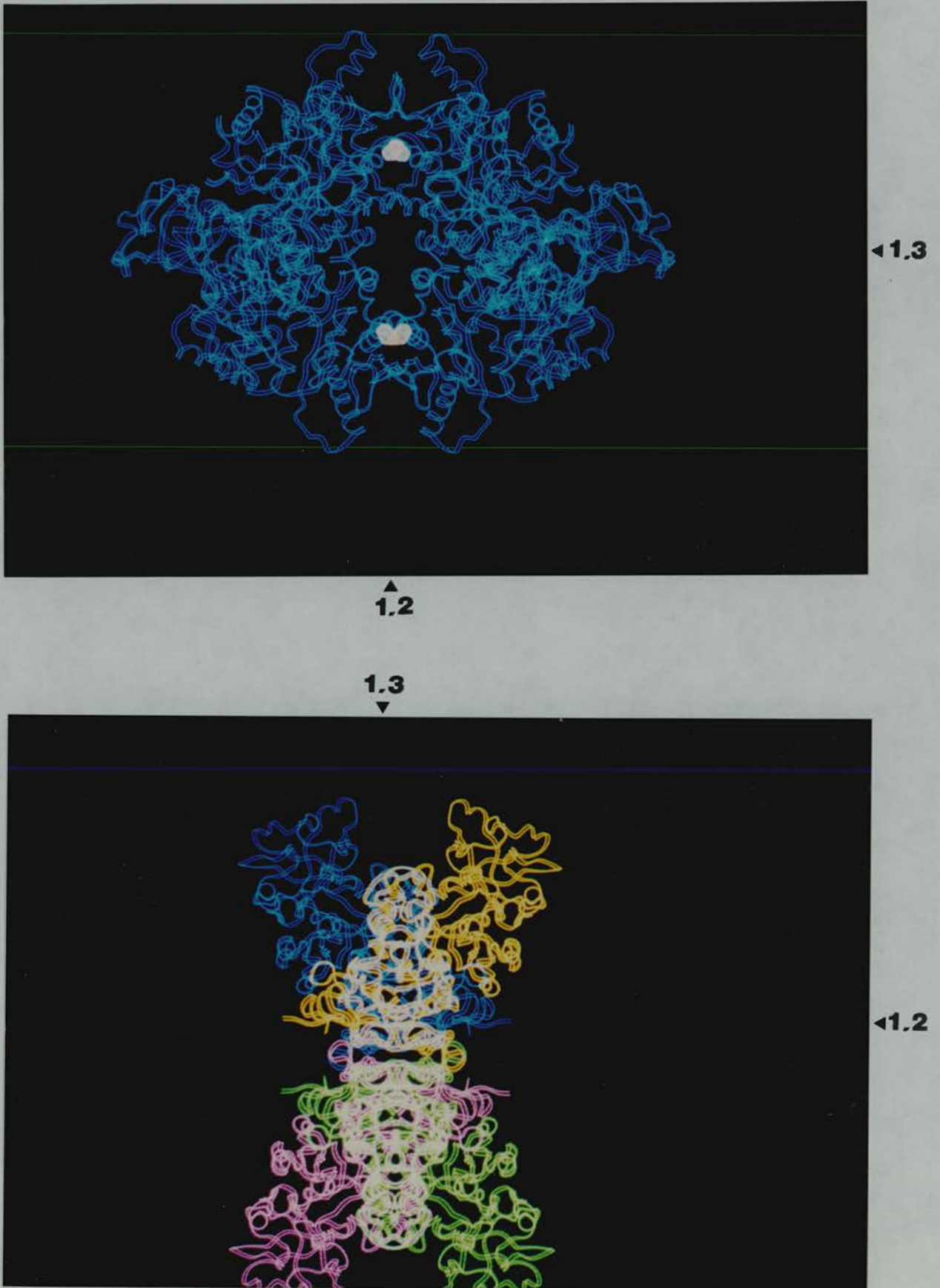


Figure 7; Tetramer of pyruvate kinase illustrating the two different types of intersubunit interface.

factors may yield better crystals of the enzyme, which will hopefully be suitable for diffraction studies.

Attempts to make site-directed mutants of pyruvate kinase without the aid of this structural model have been reported (Imarai, *et al.*, 1985). The authors do not report how they chose which residues to mutate however it is known that they had no access to the structural models described here. The residues mutated were presumably chosen on the basis of their effect on catalysis after reaction of the protein with chemically modifying reagents, however this is not always a good way to identify residues directly involved in catalysis as many residues which react with such reagents are simply surface accessible and located at or near the substrate or effector binding sites. The mutations K337Q, L and R, which involved changing the wild type lysine at position 337 to a glutamine, a leucine and an arginine residue were synthesised and expressed in an attempt to attest the role of this lysine residue as being essential to catalysis. Crude extracts are reported to show that mutants have similar levels of activity to wild type controls, thus demonstrating that this is not the essential catalytic residue. Examination of the structural model reveals that this residue is not located at the active site of the protein, but in the substrate binding site. Thus, this model proved invaluable for the design of mutations in so far as it permitted the identification of residues more likely to be involved in catalysis and regulation of the enzyme.

#### 1.1.5. Comparison of allosteric and non-allosteric forms of pyruvate kinase

It has recently been shown that the allosterically regulated mammalian M2 isoenzyme is encoded by the same structural gene as the M1 isoenzyme which displays hyperbolic kinetics under physiological conditions (Noguchi *et al.*, 1986). This gene contains 12 exons with two slightly different copies of exon 9



(fig 8). The gene is differentially spliced in a tissue specific manner which remains poorly characterised. Exon 9 encodes a stretch of 54 amino acids of which 33 remain identical between the two copies of exon 9, these translate to give two  $\alpha$ -helices designated C $\alpha$ 1 and C $\alpha$ 2 as they reside in the C-domain of the protein. X-ray crystallographic studies reveal that these helices come together to form the 1,3 intersubunit interface of the protein. Differential splicing has been categorised into 8 different classes (Breitbart *et al.* 1987). The pyruvate kinase system is an example of class 2, exon swapping. This is reported to be very common in myosal cells and has hitherto only been observed for the genes for the contractile proteins such as tropomyosin.

As the isoenzymes differ only in the region of this intersubunit contact it seems reasonable to speculate that this is the region of the protein responsible for conferring the allosteric properties of the enzyme.

#### 1.1.6. The structure of yeast pyruvate kinase

So far, attempts at determining the crystal structure of the pyruvate kinase from yeast have failed, as discussed in section 1.1.4. Pyruvate kinase is a highly conserved protein, the yeast enzyme being 65% identical to mammalian forms at the amino acid level. The three dimensional structures are generally considered to be similar and the evidence for this is borne out by the fact that it is reasonably easy to model the primary structure of the yeast enzyme using the cat M1 isoenzyme structural coordinates, the dimensions and positions of the major elements of secondary structure are well conserved between these two forms of the enzyme. The only region where the yeast enzyme is observed to be grossly different in structure to the cat M1 form is at the N-terminus. N-termini tend to be the least conserved areas of protein structure. The yeast N-terminus is much shorter than that of the cat M1

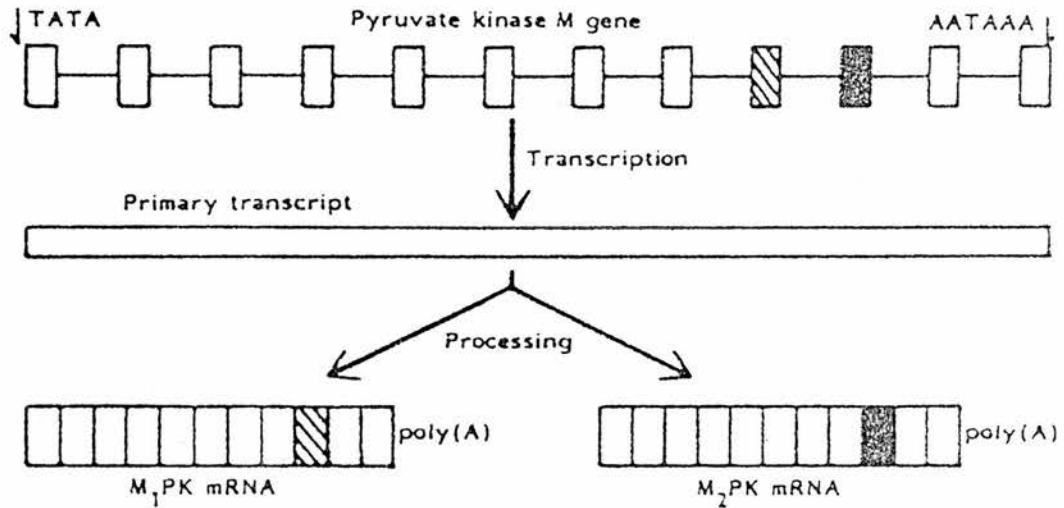


FIG. 8. Schematic representation of expression of the pyruvate kinase M gene. The M<sub>1</sub> exon is indicated by the *hatched box* and the M<sub>2</sub> exon by the *closed box*. The exons common to both mRNAs are shown by *open boxes*. TATA, TATA box; AATAAA, polyadenylation signal; M<sub>1</sub>PK, M<sub>1</sub>-type pyruvate kinase; M<sub>2</sub>PK, M<sub>2</sub>-type pyruvate kinase.

isoenzyme and this truncation would appear to take the form of a missing loop (fig 3a,b). It would be interesting to examine the role of the N-terminus in catalysis and in the regulation of pyruvate kinase. This region of the protein is strongly implicated in the control of enzyme activity since it harbours the serine residue which, in the liver isoenzyme, is phosphorylated under the control of cAMP-dependant protein kinase. This phosphorylation radically alters the catalytic properties of the enzyme as previously reported in section 1.1.3.

#### 1.1.7. The advantages of studying yeast pyruvate kinase

The pyruvate kinase from *Saccharomyces cerevisiae* has been chosen for these studies for a number of reasons. Firstly, it was anticipated that overexpression of the enzyme in a homologous system would be straightforward and could be achieved simply by transforming the host strain with a multicopy plasmid vector bearing the structural gene. Genetic manipulation of *Saccharomyces cerevisiae* is now almost as straightforward as that in *E.coli* and it is possible to disrupt and delete genes to alter the phenotypes and genotypes of host strains so that they are tailored to experimental requirements (Lundblad, 1989). A number of glycolytic enzymes were already known to be overexpressed in *S.cerevisiae*, the most noteworthy of these being phosphoglycerate kinase (PGK) (Wilson *et al.* 1986) which, when introduced into the yeast in a multicopy shuttle vector, produces around 80% of the total cell protein as PGK thus making its subsequent purification and kinetic analysis much more straightforward than from a non-overexpressing strain.

#### 1.1.8. The catalytic activity of yeast pyruvate kinase

The enzyme pyruvate kinase can catalyse a number of different reactions in

addition to that described in the opening section. These include a fluorokinase reaction in which ATP is used to convert fluoride into phosphofluoride using bicarbonate, magnesium and potassium (Teitz and Ochoa, 1958), a hydroxylamine kinase reaction whereby ATP is used to convert hydroxylamine to phosphoamine using the same effectors as above (Kupiecki and Coon, 1959), a glycolate phosphorylase reaction (Kayne, 1974) and an oxaloacetate decarboxylase reaction (Noce and Utter, 1975). While it must be noted that the substrate specificity of the enzyme is very variable, these reactions were all examined *in vitro* and are not likely to be physiological.

The reaction catalysed by pyruvate kinase is essentially an acid base reaction which occurs in two stages, the first being the transfer of the phospho group of PEP to MgADP to yield an enolate intermediate and the second being the stereo- specific protonation of this intermediate to give pyruvate (Rose, 1960). Phosphotransfer is known to take place by two different mechanisms:

1. DISSOCIATIVE The leaving group is eliminated to yield an unstable metaphosphate intermediate followed by the rapid addition of the nucleophile.
2. ASSOCIATIVE The nucleophile is added first such that a pentacovalent intermediate is formed followed by the elimination of the leaving group.

The reaction mechanism of pyruvate kinase has been extensively studied and is considered to proceed by an in line associative mechanism with nucleophilic attack on the phosphate to generate a trigonal bipyramidal intermediate and subsequent inversion of configuration (Mildvan *et al*, 1976, Hassett *et al*, 1982).

The catalytic base in this reaction is considered to be the side chain of lysine 269 which lies very close to the site of phospho-transfer. The evidence from this comes both from crystallography and from the study of the pH

profiles of the enzyme. A group with a pK of 8.3 has been found to be the acid-base catalyst implicating either a lysine or a histidine residue. (Dougherty and Clelland, 1985) The only such residue within a van der Waals distance of PEP is lysine 269 (Muirhead *et al.*, 1987). Crystallographic evidence also suggests that the side chain of this lysine may move when phosphoenolpyruvate binds to the enzyme (Clayden, 1988). The most recent line of evidence for an active site lysine comes from Electron Spin Echo Envelope Modulation studies of the enzyme with  $\text{VO}^{2+}$  (Tipton *et al.*, 1989). These workers suggest that the catalytic lysine acts as the catalytic base to mediate proton transfer from the solvent to phosphoenolpyruvate.

Pyruvate kinase requires a second magnesium ion in addition to that complexed with the nucleotide, ADP. Magnesium can be substituted by manganese and chromium, manganese being identified as a preferential activator of the enzyme although magnesium is the ion observed *in vivo* (Kayne, 1973). A number of independent lines of evidence exist to demonstrate that a unique binding site exists on the enzyme for this magnesium ion (Gupta *et al.*, 1976), Baek and Nowak, 1982). The role of the magnesium ion has recently been ascribed as coordinating the phosphoryl group as it is transferred from phosphoenolpyruvate to MgADP during catalytic turnover (Tipton *et al.*, 1989). This ion is also reported to be a ligand for the enolate leaving group. The enolate is considered to be a very poor leaving group in the absence of the enzyme bound magnesium.

The essential potassium ion has also been studied by NMR spectroscopy. This ion can be substituted by a number of other monovalent cations, the maximal activity of the enzyme with  $\text{K}^+$  being a function of the ionic radius of the cation (Villafranca and Raushel, 1982). The potassium ion is thought to play a role in increasing the specificity of the enzyme for phosphoenolpyruvate by

increasing the enzyme affinity for phosphoenolpyruvate and decreasing the affinity for analogues and potential substrates where the carboxyl group is missing (Nowak and Mildvan, 1972).

The nucleotide bound magnesium serves to reduce the electrostatic repulsion between the phosphodonor, phosphoenolpyruvate, and the nucleophile, enolate. It also helps to position the  $\alpha$  and  $\beta$  phospho groups in the active site. The nucleotide bound magnesium also performs these functions in other kinases, e.g. phosphoglycerate kinase (Blake and Rice, 1981)

1.2.  $\alpha/\beta$  BARREL ENZYMES

Pyruvate kinase is one of the class of 17 known  $\alpha/\beta$  barrel enzymes. These are enzymes which contain an eight stranded  $\alpha/\beta$  barrel element of protein tertiary structure (Richardson, 1981). The secondary structure consists of eight  $\beta$  strands alternating with one or sometimes two  $\alpha$  helices. The chain folds so that the eight strands form a parallel  $\beta$  sheet which twists to give a closed barrel structure. The helices then pack onto the exterior of the barrel. Figure 9 shows the barrel structure of pyruvate kinase while Table 3 lists all known enzymes with this element of tertiary structure and also describes some of the common features that these enzymes share. In pyruvate kinase the element comprises only one domain of a protein monomer, domain A. In this enzyme the barrel is not a contiguous structure in that the main chain forms the first part of the barrel then folds domain B before returning to complete the closed barrel element. In the enzyme triosephosphate isomerase the barrel is contiguous and forms the single domain of the protein monomer.

The active sites of most of the enzymes in table 3 are in the same position with respect to the barrel, that is, in a cleft formed where the C-termini of the  $\beta$  strands folds around to join the N-termini of the  $\alpha$  helices (Chothia, 1988). The only exception to this would seem to be the rabbit muscle fructose-1,6-bisphosphate aldolase where the active site is located at the core of the barrel, a region which is uncharged in all other enzymes of this class (Sygusch *et al.*, 1987, Gamblin *et al.*, 1990). Presumably the fructose-1,6-bisphosphate aldolase has evolved a charged barrel core to bind the highly negatively charged substrates of the reaction. Yeast enolase, while first reported to be a member of this class of enzyme (Lebioda and Stec, 1988) is now considered to have an  $\beta\beta\alpha\alpha(\beta\alpha_6)$  structure, that is to say that the  $\beta$  strands are not all parallel, one is oriented antiparallel to the others while one



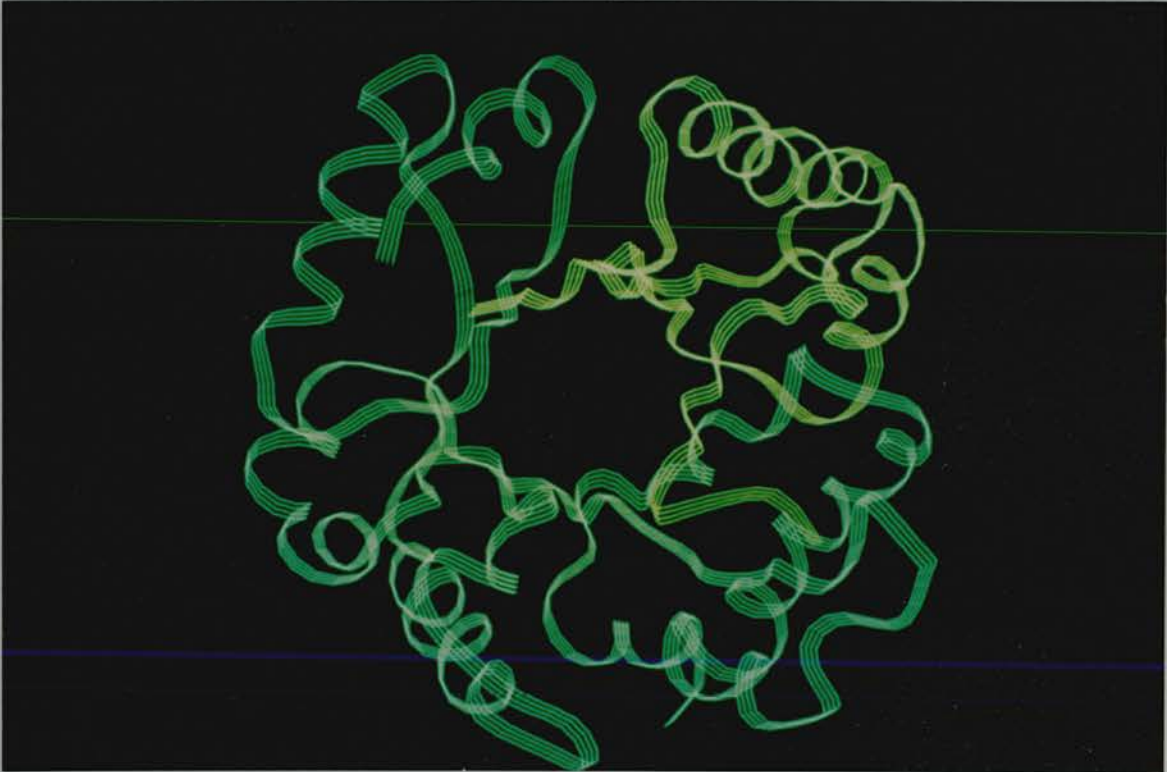


Figure 9: The  $\alpha/\beta$  barrel of pyruvate kinase.



Table 3; All known  $\alpha/\beta$  barrel enzymes.

ENZYME	REACTION	COFACTOR
PYRUVATE KINASE [EC. 2.7.1.40]	ADP + Mg <sup>2+</sup> + PEP ATP + PYRUVATE + H <sup>+</sup>	Mg <sup>2+</sup>
TAKA AMYLASE A [EC. 3.2.1.1]	ENDOMYOLYTIC DEGRADATION OF STARCH	Ca <sup>2+</sup>
RIBULOSE 1,5 BISPHOSPHATE CARBOXYLASE/ OXYGENASE [EC. 4.1.1.30]	D-RIBULOSE 1,5 BISPHOSPHATE + CO <sub>2</sub> = 2,3 BISPHOSPHOGLYCERATE	Cu <sup>2+</sup>
GLYCOLATE OXIDASE [EC. 1.1.3.1]	GLYCOLATE + O <sub>2</sub> = GLYOXYLATE + H <sub>2</sub> O <sub>2</sub>	FMN
D XYLOSE ISOMERASE [EC. 5.3.1.5]	D XYLOSE = D XYLULOSE	Mg <sup>2+</sup>
TRIOSEPHOSPHATE ISOMERASE [EC. 5.3.1.1]	D-GLYCERALDEHYDE-3-PHOSPHATE = DIHYDROXYACETONEPHOSPHATE	-
TRYPTOPHAN SYNTHASE [EC. 4.2.1.20]	L-SERINE + INDOLYLGLYCERO- PHOSPHATE = L-TRYPTOPHAN + GLYCERALDEHYDE	-
KDPG ALDOLASE [EC. 4.1.2.14]	6-PHOSPHO-2-KETO-3- DEOXYGLUCONATE = PYRUVATE + D-GLYCERALDEHYDE-3-PHOSPHATE	-
FRUCTOSE-1,6-BIS PHOSPHATE ALDOLASE [EC. 4.1.2.13]	FRUCTOSE-1,6-BISPHOSPHATE = D-GLYCERALDEHYDE-3-PHOSPHATE + DIHYDROXYACETONE PHOSPHATE	-
TRIMETHYLAMINE DEHYDROGENASE [EC. 1.5.99.7]	TRIMETHYLAMINE + H <sub>2</sub> O + ACCEPTOR = DIMETHYLAMINE + FORMALDEHYDE + REDUCED ACCEPTOR	FMN IRON/SULPHUR
N-(5PHOSPHORYBOSYL) ANTHRANYLATE ISOMERASE/ INDOLE-3-GLYCERO PHOSPHATE SYNTHASE [EC.2.4.2.18] & [EC. 4.1.1.48]	PRA = CdRP CdRP = IGP + H <sub>2</sub> O + CO <sub>2</sub>	-
MUCONATE LACTONISING ENZYME [EC. 5.5.1.1]	(+)-4-CARBOXYMETHYL-4- HYDROXYCROTONOLACTONE = CIS CIS MUCONATE	Mn <sup>2+</sup>
FLAVOCYTOCHROME B <sub>2</sub> [EC. 1.1.2.3]	L-LACTATE + 2 FERRICYTOCHROME B <sub>2</sub> = PYRUVATE + 2FERRICYTOCHROME B <sub>2</sub>	FMN HAEM
ENOLASE [EC.4.2.1.11]	2 PHOSPHO-D-GLYCERATE= PHOSPHOENOLPYRUVATE + H <sub>2</sub> O	Mg <sup>2+</sup>
MANDELATE RACEMASE [EC.5.1.2.2]	S-MANDELATE= R-MANDELATE	Mg <sup>2+</sup>
CYCLODEXTRIN GLYCOSYLTRANSFERASE [EC.2.4.1.19]	1,4 α D-GLUCOPYRANOSE (Gn) = Gn-x + CYCLODEXTRIN	Ca <sup>2+</sup>

CATALYTIC RESIDUE	SOURCE	QUATERNARY STRUCTURE	REFERENCE
LYS	CAT MUSCLE	TETRAMER	Stuart ( <i>et al.</i> ) (1979)
ASP	<i>Aspergillus oryzae</i>	MONOMER	Matsuura ( <i>et al.</i> ) (1984)
LYS	<i>Rhodospirillum rubrum</i>	MULTISUBUNIT	Schneider ( <i>et al.</i> ) (1986)
N/A	SPINACH	TETRAMER	Lindqvist and Branden (1985)
-	<i>Streptomyces rubiginosus</i>	DIMERS AND TETRAMERS	Carrel ( <i>et al.</i> ) (1984)
LYS	CHICKEN MUSCLE	DIMER	Banner ( <i>et al.</i> ) (1979)
GLU	<i>Salmonella typhimurium</i>	-	Hyde ( <i>et al.</i> ) (1987)
LYS	<i>Pseudomonas putida</i>	TRIMER	Mavridis ( <i>et al.</i> ) (1982)
-	RABBIT MUSCLE	TETRAMER	Gamblin ( <i>et al.</i> ) (1990)
N/A	Methylophilic bacterium W <sub>3</sub> A	DIMERS	Lim ( <i>et al.</i> ) (1986)
-	<i>Eschericia coli</i>	MONOMERIC BIFUNCTIONAL	Preistle ( <i>et al.</i> ) (1987)
-	<i>Pseudomonas putida</i>	OCTAMER	Goldman ( <i>et al.</i> ) (1987)
N/A	<i>Saccharomyces cerevisiae</i>	TETRAMERS	Xia ( <i>et al.</i> ) (1987)
LYS GLU	<i>Saccharomyces cerevisiae</i>	DIMER	Lebicda and Stec (1988)
-	-	-	Faber and Petsko (1990)
-	-	-	Hoffman ( <i>et al.</i> ) (1990)

of the  $\alpha$  helices is also reversed (Lebioda and Stec, 1989). However the structure would seem to be sufficiently similar to merit its inclusion in this list of enzymes.

The eight stranded barrel is a very interesting structure from an evolutionary standpoint and contention rages in the literature as to whether the structural element arose by a process of convergent evolution to a particularly stable element, or by divergent evolution from a common ancestor (Levine *et al.*, 1978, Lebioda *et al.*, 1982, Chothia, 1988, Lebioda and Stec, 1988). There are three main lines of evidence which can suggest that enzymes have arisen from a common ancestor, these are sequence similarities, structural similarities and geometry of the active sites (Chothia, 1988). There is no doubt that these enzymes are strongly structurally similar and in most cases the active site geometries are identical, however there is no identifiable sequence similarity between any of the  $\alpha/\beta$  barrel enzymes (Fothergill-Gilmore, 1986).

There is also a mathematical formula reported to determine the evolutionary relatedness of any two structurally similar enzymes, derived from empirical observations.

$$\Delta = 0.40e^{1.87H}$$

where  $\Delta$  is the rms deviation of main chain atoms when the two similar structures are superimposed, and H is the proportion of non-identical amino acid residues (Chothia and Lesk, 1986). Since both of these criteria are readily measurable, it is possible to feed them into the equation and, if both sides of the equation are equal then the proteins can be said to have evolved from a common ancestor. It would be very interesting to examine the relatedness of the glycolytic enzymes with this structural motif. The fact that there are four glycolytic enzymes with this common fold may be indicative of divergent evolution from a common ancestor. This may not be surprising in the case of

enolase and pyruvate kinase which catalyse sequential reactions in the glycolytic pathway. Further evidence for the fact that they have evolved divergently comes from the fact that they both require both a monovalent and a divalent cation for full enzyme activity.

Recently an  $\alpha/\beta$  barrel protein has been synthesised de novo (Goraj *et al.*, 1990). This was done by assuming that the structure was an 8 fold repeat of four elementary structural features, a turn, a  $\beta$  strand, another turn followed by an  $\alpha$  helix. Plasmids were manufactured so that they could produce 7, 8, or 9 of these elements in tandem, in an *E. coli* cell. These proteins were referred to as heptarellin, octarellin and nonarellin. Urea gradient gel electrophoresis studies suggest that only octarellin can refold into a compact structure after denaturation. This refolding is reported to be a cooperative two state transition. It is tempting to use this in vitro analysis as weight to an argument for convergent evolution to a stable folded structure. Further folding studies on the  $\alpha/\beta$  barrels have been published recently, which demonstrate the stability of the eight stranded barrel (Luger *et al.*, 1990). The case for convergent evolution is more difficult to argue as there can be no ancestral evidence, as in the case for divergence. It has been suggested that, due to constraints governing protein folding, proteins may only be able to adopt a finite number of conformations. These constraints are thermodynamic and kinetic and are determined largely by the primary structure of a protein (Finklestein and Ptitskyn, 1987). In that case the eight stranded  $\alpha/\beta$  barrel may simply represent the most stably folded structure for these proteins. Further evidence for convergent evolution comes from the recent discovery that these enzymes may be divided into two subtypes depending on the packing of the interior of the sheet (Lesk *et al.*, 1989). These two classes of enzyme could not interconvert without gross rearrangement of the active site, and since one of the shared properties of all but one of these enzymes is the position of the

active site it seems unlikely that these have emerged divergently unless it was from two different ancestral molecules. The most compelling evidence for convergent evolution is the total lack of any sequence similarity between the enzymes which share this structural element, however it is argued that this is because divergence occurred so long ago that it is no longer observable (Fothergill-Gilmore, 1986). It is generally accepted that bifunctional enzymes arise from a gene duplication event, both parts of the enzyme then evolving to catalyse its specific reaction. In the case of N-(5'phosphoribosyl) anthranilate isomerase/indole-3-glycerolphosphate synthase, the separate domains have only 10% sequence identity demonstrating that sequence identity is rapidly lost during the evolutionary process (Prestle *et al.*, 1987).

The most recent review on the evolution of the  $\alpha/\beta$  barrel proteins (Faber and Petsko, 1990) suggests that these enzymes have diverged from a common ancestor. The authors state that all of the 17 known  $\alpha/\beta$  barrel proteins are enzymes. While this is indeed true, some of the enzymes have other biological functions. Enolase is also found as a crystallin in reptile eye lens (Wistowge & Pitigorsky, 1987) and monomeric pyruvate kinase is also reported to be a cytosolic thyroid hormone binding protein in humans (Kato *et al.*, 1989). It is not therefore strictly true to say that all of the proteins with the eight stranded  $\alpha/\beta$  barrel motif have a catalytic function. In this review, a strong case for divergent evolution is implied from the fact that all of the active sites are located at the C-terminal portion of the barrel. While this is indeed true, almost all of the substrates for these enzymes are highly negatively charged, and binding to this face of the barrel is favoured by the helix dipole and the charge distribution across the barrel. The authors then proceed to divide the enzymes up into four broad structural families on the basis of the crystallographic data, although they acknowledge that these crude structural divisions are not conclusive proof of a common ancestor.

It is tempting to speculate that  $\alpha/\beta$  barrel enzymes first evolved to contain a prosthetic group to facilitate catalysis such as flavin and 4Fe/4S in the case of the trimethylamine dehydrogenase of the methylotrophic bacterium *W<sub>3</sub>A* (Lim *et al.*, 1986), then evolved to be able to undergo catalysis without the prosthetic group. All of these enzymes catalyse reactions with planar substrates, perhaps a reflection of the primordial cofactor. In this scenario the most highly evolved enzymes would be those which catalysed a reaction with a substrate which was not perfectly planar. Clearly it is impossible to do anything but speculate on whether the eight stranded  $\alpha/\beta$  barrel emerged by a process of convergent or divergent evolution. As new structures are elucidated, perhaps new evidence may emerge. An interesting insight may be gained by close studies of the archaeobacterial counterparts of these 17 structurally solved proteins. Crystal structures of a few of these ancestral proteins might give evidence of how the structures evolved.

### 1.3. SITE DIRECTED MUTAGENESIS

Site directed mutagenesis (SDM) is a reasonably new technology, initial protocols for the technique being published as recently as 1982 (Zoller and Smith, 1982). Initial experimental approaches involved the use of the bacteriophage M13 (Messing *et al.*, 1977) as a source of single stranded DNA template to which a mismatch oligonucleotide was hybridised and a second strand synthesised in vitro using the mutagenic oligonucleotide as a primer. This hybrid was then used directly for transformation of *E. coli* and the progeny plaques screened by differential hybridisation with the mutagenic oligonucleotide. In theory this method of SDM should be around 50% efficient, in practise however, a much higher background of wild-type phage are obtained and efficiencies of 1 - 3% are more commonly obtained. Within only four years of the publication of these initial methods the technology has improved such that efficiencies of 80 - 100% are routinely obtained. This precludes the requirement for extensive screening since a few phage plaques can be isolated and grown for DNA sequencing directly, without any primary screening. Other advances in this methodology make it possible to replace any amino acid, in a site specific manner, with each of the 19 others in only a few steps, either by cassette mutagenesis (Wells *et al.*, 1985), or by using nonsense suppressing tRNAs (Rossi and Zoller, 1987). Site directed mutagenesis need not only be used to specifically change one amino acid residue. Oligonucleotides may also be used to generate insertions or deletions in a DNA strand and a single oligonucleotide can be deigned to alter any number of amino acid at any one time. The applications of SDM are only as limited as the researchers imagination. The value in relation to the understanding of the reaction mechanism of pyruvate kinase and its regulation by allosteric effectors will be discussed in this thesis. SDM is also used as a tool for the study of the



mechanism of protein folding (Ackers and Smith, 1985), the understanding of protein stability (Wells, 1987), the production of specialised translation systems (Hui and de Boer, 1987) and protein/protein recognition (Staton *et al.*, 1988) to cite only a few of the many examples.

### 1.3.1. SDM METHODS

The two methods of SDM most widely used are those of Kunkel (1985) and Eckstein (1985), both of these methods are in use in our laboratory and are highly efficient. The Eckstein method is supplied in kit form and relies on the use of a synthetic dCTP analogue, dCTP $\alpha$ S, which makes the method fairly costly since this analogue is not widely available. dCTP $\alpha$ S is incorporated into a DNA duplex using an M13 single strand template and a mutagenic oligonucleotide as a primer for second strand synthesis. This duplex is no longer a substrate for certain classes of restriction endonuclease such that they no longer cut the DNA at the recognition site where the analogue is present, but produce a nick in the parent DNA strand. This nicked duplex is then used as a substrate for limited digestion with EXO III so that the non-mutated area of the duplex is removed. The duplex is repaired *in vitro* with DNA polymerase and T4 DNA ligase and transformed into wild-type *E.coli* cells. This process yields around 95% mutant plaques according to the manufacturers instructions.

The Kunkel method utilises a strain of *E.coli* with a *dut<sup>-</sup>ung<sup>-</sup>* genotype, deficient in the enzymes dUTPase and uracil-N-glycosylase, thus there is an increased intracellular pool of dUTP such that dUTP is incorporated into the DNA more frequently. The *ung* phenotype prevents this dUTP from being excised from the DNA by the usual repair mechanisms. Phage DNA propagated from such a strain, when transformed into a (*dut<sup>+</sup>ung<sup>+</sup>*) strain, will no longer

form viable progeny. If single stranded DNA from these phage is used as a template for SDM and a second strand is formed *in vitro* using a mismatch oligonucleotide as a primer for second strand synthesis, then this heteroduplex used to transform  $\text{dut}^+$ ,  $\text{ung}^+$  host cells, only the mutant strand will form viable progeny thus increasing the efficiency of the mutagenesis. Figure 10 illustrates the sequence of reactions in the Kunkel mutagenesis method, this was the method employed throughout this project and in my hands this turned out to be 80-100% efficient.

### 1.3.2. THE MUTAGENESIS REACTION

Both of the standard mutagenesis methods require that the gene to be mutated should be in a single stranded form. This is usually achieved by cloning the gene of interest into a filamentous phage vector such as M13 (Messing *et al.*, 1976).

The disadvantage of this system is that, although in theory inserts in the M13 vector can be of any size, in practise, large inserts (> 1Kb) are unstable, as are stretches of DNA with extensive secondary structure (Zinder *et al.*, 1982). Attempts to clone the entire pyruvate kinase coding gene into M13 proved unsuccessful, so a small fragment of the coding region was cloned and mutated. Extensive subcloning was then required to produce the entire coding sequence of the gene in an *S. cerevisiae*/*E. coli* shuttle plasmid for expression of the mutant proteins.

One of the principal requirements of SDM is accurate DNA sequence data, since it is from this information that mutagenic oligonucleotides are designed. The gene for pyruvate kinase from *S. cerevisiae* had been cloned and its DNA sequence determined prior to the onset of this project (Burke *et al.*, 1983). Unfortunately there were errors in this published sequence which caused

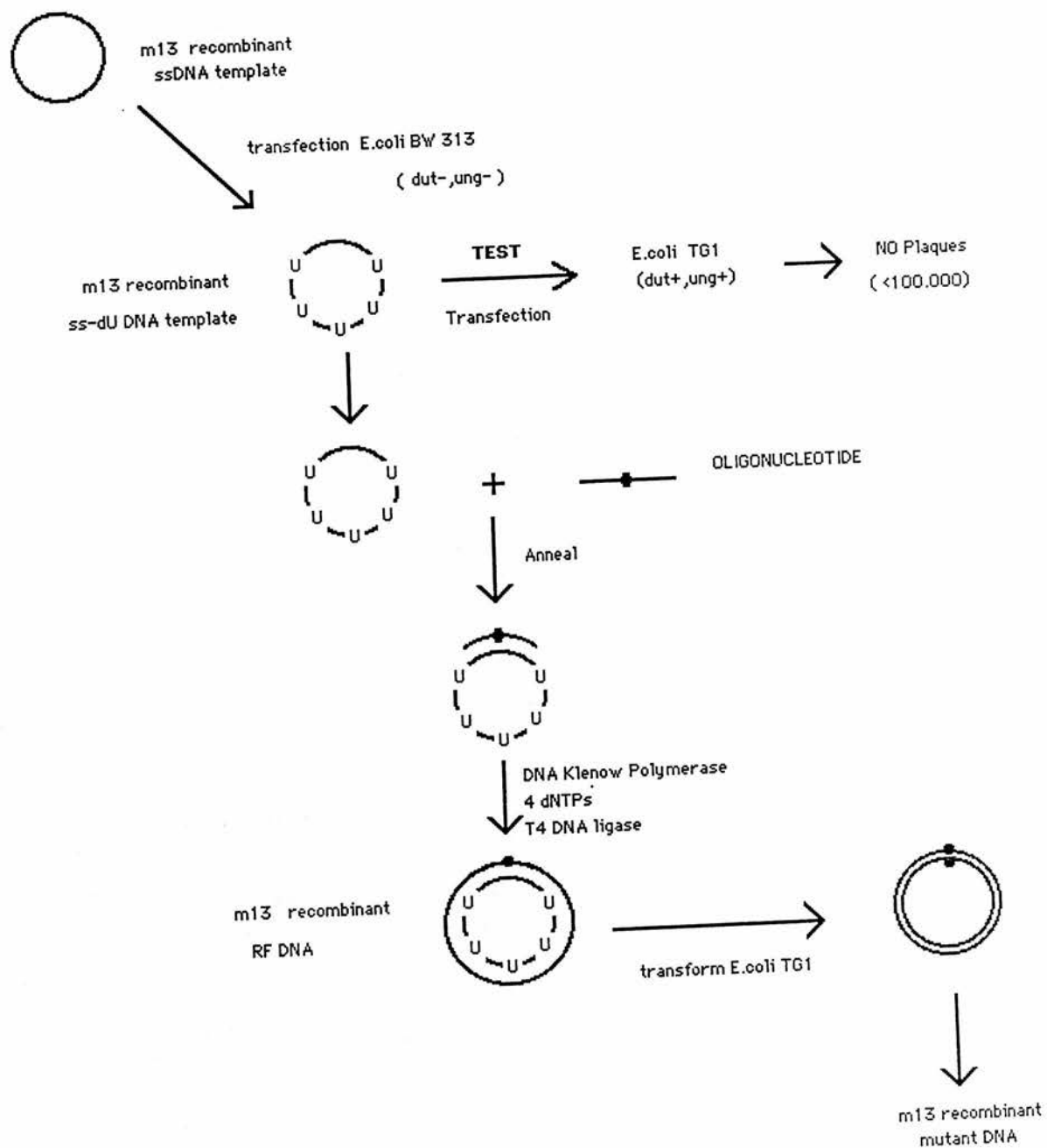


Figure 10; The Kunkel method of site directed mutagenesis.

considerable delay to this mutagenesis project. The errors were located within the region of the gene encoding the C $\alpha$ 1 and C $\alpha$ 2 helices. Initial attempts to make mutants in this region all failed as the oligonucleotides were designed to match the wrong sequence and so did not bind to the DNA at the points predicted. This region of the gene is highly GC rich and the sequencing errors are presumed to be a result of GC stacking in the sequencing gel. The methods employed to alleviate this problem will be discussed. The gene has now been completely resequenced and these errors corrected. These were the only errors found in the entire gene.

### 1.3.3. DESIGN OF OLIGONUCLEOTIDES

The technique of SDM relies on the priming of a mismatch oligonucleotide to a specific site on a gene, which drives the mutagenesis reaction and is the basis of the site specificity. It is necessary to invest some careful thought into the design of these oligonucleotides. Mutagenic oligonucleotides tend to be around 18–20 bases in length so as to form a stable hybrid with the single stranded template, thus allowing priming from the 3' end of the oligonucleotide. The mismatch should be located slightly toward the 5' end of the oligonucleotide, this again encourages a more stable 3' priming site. Optimally the oligonucleotide should be as GC rich as possible to increase the binding strength, however this will be largely determined by the sequence of the DNA around the oligonucleotide binding site. It is particularly useful to have a G or C residue at each end of the oligonucleotide wherever possible. It is useful, too, to run the sequence of the oligonucleotide through a sequence matching programme, such as GAP in the UWGCG package to ensure that it has no significant identity with the sequence of M13. If significant matching does occur, this may direct a mutation within the M13 genome which, if deleterious, may abolish phage replication.

In these experiments oligonucleotides were designed bearing in mind the codon bias observed in *S. cerevisiae* (Bennetzen and Hall, 1983). Once an oligonucleotide has been designed its effectiveness can be ascertained by using it as a primer in a DNA sequencing reaction. Generally oligonucleotides which prime a good, clean DNA sequence will make good mutagenic oligonucleotides.

Once a mutant has been made it is necessary to design a system to effect its characterisation.

#### 1.4. OVEREXPRESSION OF PYRUVATE KINASE IN *SACCHAROMYCES CEREVISIAE*

The pyruvate kinase gene (*pyk1*) from *Saccharomyces cerevisiae* has been cloned by complementation (Kawasaki and Fraenkel, 1982), and the DNA sequence determined (Burke *et al.*, 1983, McNally *et al.*, 1989). The *pyk1* gene is known to complement the *cdc19* mutation in *S. cerevisiae*, a cell division cycle mutation. This means that in the absence of a viable pyruvate kinase gene, the cells fail to divide. It is not clear what relationship exists between pyruvate kinase expression and cell division. If the two are interrelated one might anticipate some difficulty in engineering yeast cells to overexpress pyruvate kinase.

In general, the transformation of a yeast gene into yeast using a multicopy (2 $\mu$  based) plasmid vector, leads to the synthesis of high levels of the gene product. This does not appear to be true for pyruvate kinase when under the control of its own promoter, for although newly transformed strains overexpress pyruvate kinase approximately 12-fold, the level of overexpression falls back to wild type levels during continued culture on selective media, or on storage on selective plates at 4°C. This phenomenon has been observed in other laboratories (Al Brown and Toby Murcott, personal communications). The over-expressing strain grows more slowly than wild-type, the growth rate increasing as the level of overexpression decreases, suggesting that overexpression of pyruvate kinase is deleterious to the cell.

The *pyk1* gene is subject to dosage compensation (Moore *et al.* 1990a.) This effect is observed both at the level of transcription and translation. That is to say that, at the transcriptional level, in the presence of multiple copies of the *pyk1* gene the number of copies of mRNA produced from each gene is reduced. At a translational level, excess copies of *pyk1* mRNA are translated

less efficiently than basal levels. This phenomenon has so far been restricted to gene families encoding histone and ribosomal proteins (Osley and Hereford, 1981, Warner *et al.*, 1985). Defects in these genes can also give rise to *cdc* mutations.

The dosage compensation effect is not observed with any other glycolytic genes, indeed phosphoglycerate kinase, when overexpressed can comprise 50–80% of the total cell protein under certain conditions (Mellor *et al.*, 1985) with no apparent deleterious effect on the cells. Attempts have been made to overexpress all of the glycolytic genes homologously in *S.cerevisiae* (Schaff *et al.*, 1989). Increased levels of expression were obtained for all of the enzymes tested. The overproduction of pyruvate kinase and phosphofructokinase was coupled by having the genes *pyk1*, *pfk1* and *pfk2* present on the same multicopy plasmid. An increase in the activities of both of these enzymes led to a decrease in cell growth rate. There are no reports of such studies using the *pfk* genes alone. It has recently been demonstrated that the translation of *pfk* mRNA is severely inhibited in the presence of high *pyk1* mRNA levels suggesting that the dosage compensation effect operates in trans, (Moore *et al.* 1990b)

Activities of several enzymes involved in carbohydrate metabolism have been measured during the cell cycle of *S.cerevisiae* (Van Doorn *et al.*, 1988). Pyruvate kinase levels are seen to increase markedly during the G2 phase of the cycle demonstrating that the levels of pyruvate kinase expression are subject to some form of regulation during cell division. This periodicity was also observed with hexokinase and phosphofructokinase, although neither of these alleles complement *cdc* mutations. The precise nature of this periodicity has not yet been determined. This form of regulation may reflect the fact that, as ATP utilising enzymes, these are considered to be the rate controlling steps



for the glycolytic pathway, so their levels may be decreased in order to reduce flux through glycolysis.

When the *pyk1* gene is transformed into *S.cerevisiae* under the control of the *pgk* promoter, one of the strongest yeast promoters known, the levels of pyruvate kinase protein produced are very high, around 20% of the total cell protein. These high levels of protein production are maintained throughout cell culture and storage and would seem to be of little or no disadvantage to the cells (this work and J.Mellor, personal communication.) From this evidence it would appear that the upstream control elements mediate the level of expression. Upstream secondary structure formation is reported to reduce the levels of translation of *pyk* mRNA (Bettany *et al.*, 1989) but this could not account for the reduction in the concentration of mRNA observed after prolonged growth or storage.

An upstream activating sequence and upstream repressible sequence have been identified upstream of the *pyk1* gene (Nishazawa *et al.*, 1989). These sequences are reported to comprise a mechanism for carbon source dependant regulation of pyruvate kinase. Such cis-activating regulatory factors, when present in high copy number, may dilute out some factor, as yet uncharacterised, which is required for transcriptional regulation. During periods of prolonged growth or storage production of this factor may be permitted to increase such that it can exert a measurable effect on the transcription of the *pyk1* gene.

The *pgk* promoter has recently been shown to bind the product of the *rap* gene, and *rap* is reported to activate transcription (Chambers *et al.*, 1989). The *rap* binding site has been identified and is similar, though not identical to the upstream activator sequence reported in the *pyk1* upstream region (Nishazawa *et al.*, 1989). In this same publication there is also reported to be an upstream

repressible sequence in *pyk1*. Such an upstream repressible sequence is also reported in the ribosomal protein genes, which are also subject to dosage compensation. It is not clear whether is related to the dosage compensation phenomenon. However it is clear that dilution of a repressor protein by overproduction of the site to which it binds could not give rise to the dosage compensation effect which manifests itself as a decrease in protein production.

The products of the hexokinase reaction are reported to control the synthesis of the glycolytic enzymes, among these is pyruvate kinase (Maitra and Lobo, 1971), however it was not clear at the time of their publication whether this arose at the level of transcription or translation. This mechanism presumably plays little or no effect on the inability to overexpress *pyk1* under the control of its own promoter since this induction is common to all the glycolytic enzyme genes.

In all of the experiments where *pyk1* was expressed under the control of its own promoter, a large upstream area of the gene was maintained in the multicopy vector. This area was around 2Kb in size. The uppermost regulatory element thus far reported is at -811bp from the start codon of the gene. Sequencing studies on this area of chromosome 1 from *S.cerevisiae* show that all of the *cyc3* gene and at least part of the *fun10* gene are present on this fragment of DNA (Coleman *et al.*, 1986). One should not discount the possibility that the altered expression of *pyk1* is some hitherto unexplained consequence of the overexpression of *cyc3* or *fun10*.

Since experimental evidence strongly suggests that it is the upstream region of the *pyk1* gene which is deleterious to cell growth and not the presence of the pyruvate kinase protein itself, the following strategy for overexpression was adopted. The *pyk1* gene was obtained cloned into the expression vector pMA91 (Mellor *et al.*, 1986) such that pyruvate kinase

expression was under the control of the *pgk* promoter. This construct was used as the source of enzyme in these studies.

### 1.5. ALLOSTERIC INTERACTIONS OF PROTEINS

Pyruvate kinases are allosterically regulated with the exception of the enzyme from skeletal muscle (Hall and Cottam, 1978). Allosteric regulation of proteins is defined as the modulation of protein activity by the binding of a molecule, which is not a substrate molecule at a site on the enzyme distinct from the substrate binding site. The binding of the allosteric effector to an enzyme may either increase or decrease its catalytic activity. The term 'allosteric' stems from the Greek 'allo' meaning other and 'stero' pertaining to shape.

Pyruvate kinase is also cooperative with respect to its substrate phosphoenol pyruvate. Cooperativity is the phenomenon by which binding of a substrate molecule to the substrate binding site facilitates the binding of further substrate molecules to the other subunits of the protein. Allostery and cooperativity are quite closely linked in that proteins frequently exhibit both of these phenomena. Some proteins are cooperative but not allosteric ie. lamprey haemoglobin (Briehl, 1963) but by far the majority are both allosteric and cooperative.

The term 'allosteric' was first coined in a report concerning cooperativity and feedback inhibition of enzymes published by Monod, Changeux and Jacob in 1963. The concept of allostery was then considered to be "the second secret of life", the first being the discovery of DNA. This original work prophesied that "more complete observations, once available, might justify the conclusion that allosteric transitions frequently involve alterations in quaternary structure." It is just such an alteration in quaternary structure which is proposed to effect the allosteric regulation of pyruvate kinase, and has been demonstrated in a large number of allosteric proteins.

Two models for allosteric control have been proposed. The first, the original by Monod, Wyman and Changeux suggest that allostery and cooperativity may arise in proteins with two or more structures in equilibrium. It predicts that such proteins are likely to be made up of numbers of subunits symmetrically arranged and that an alteration in the arrangement of these subunits or the bonds between them would occur during the allosteric transition. The model proposed two distinct structures, R and T, R being the relaxed and more active form of the enzyme and T being the tense less active form constrained by strong bonds that would resist the tertiary structural change required for effective substrate binding. In the R form such constraints are relaxed. This model is frequently referred to as the symmetry or concerted model for allosteric control and has the limitation that all substrates for the protein at any one time would be in one state.

The later model for allosteric control proposed by Koshland, Nemeny and Filmer in 1966 had no such symmetry restrictions. Each substrate is permitted to change its tertiary structure on substrate binding and thereby to affect the chemical activities of its neighbours. This is frequently referred to as the sequential model.

A much later model by Cooper and Dryden (1984) proposes allostery without conformational change suggesting that changes in entropy upon effector binding are sufficient to alter the activity of the protein.

#### 1.5.1. Haemoglobin

Haemoglobin was the first allosteric protein for which detailed stereochemical information was available since the X-ray structures of both the R and the T state were the first available at high resolution (Perutz 1978). The simplest example of all the haemoglobins is that of Lamprey (Briehl, 1963).

This protein is not regulated by allosteric effectors but is cooperative with respect to oxygen binding. The reaction with oxygen is based on a reversible dissociation into subunits. In the absence of oxygen the protein exists as dimers and tetramers with low oxygen affinity but in the presence of oxygen these dissociate into monomers with high oxygen affinity (Hendrickson and Love, 1971). While lamprey haemoglobin may not be an example of a truly allosteric protein, merely a cooperative one, it is an example of how a cooperative interaction can affect an intersubunit boundary.

Human haemoglobin however is allosterically regulated. The protein is a tetramer of two A and two B chains. Each subunit contains one haem prosthetic group. Like pyruvate kinase the protein can be considered to be a dimer of dimers, having one extensive intersubunit interface and one which has a smaller surface area and as such is regarded as more flexible. Haemoglobin is allosterically regulated by  $H^+$ ,  $CO_2$  and 2,3-bisphosphoglycerate. At low concentrations of oxygen, dissociation of tetramers into dimers can occur, an analogous situation to that found in the lamprey enzyme. However, at physiological oxygen concentrations an equilibrium exists between the deoxy T state of the protein, the tense form of the protein with low oxygen affinity and the oxy R state, the relaxed form with high oxygen affinity. The R and T forms differ in the arrangement of the subunits and in the tertiary structure within the subunits. The allosteric transition involves rotation of one pair of subunits relative to the other about the less extensive intersubunit interface. (Baldwin and Chothia, 1979). The tertiary structure of each dimer also changes to compensate for the quaternary structural changes. The allosteric effectors all exert their effects by encouraging the formation of hydrogen bonds to specifically stabilise the T state of the protein and thus encourage dissociation of oxygen from the protein (Fermi and Perutz, 1981). This review also describes a number of naturally occurring mutants of haemoglobin found in the

human population, some with altered hydrogen bonding specificity, which have facilitated the study of the allosteric transition in this protein.

### 1.5.2. Glycogen Phosphorylase

Glycogen phosphorylase *b* is reported to have been the first allosteric protein to be isolated and analysed in detail (Cori and Cori, 1936), indeed, long before the allosteric theories of Monod and Koshland had been proposed. The enzyme is regulated by both allosteric interactions and reversible phosphorylation (Graves and Wang, 1972). Phosphorylase *a* is the phosphorylated form and is the active form of the enzyme. Phosphorylase *b* is dependant on AMP for activity, and this activity is inhibited by ATP and glucose-6-phosphate, which are allosteric effectors. Phosphorylase *b* is a dimer of two identical subunits in the absence of effectors. Activation by phosphorylation or in the presence of AMP produces a change in the oligomeric state of the enzyme such that it becomes largely tetrameric, these tetramers having only 12-33% of the dimeric enzyme activity. These tetramers may be dissociated by glycogen or oligosaccharides to yield fully active dimers. (Huang and Graves, 1970) Phosphorylase *a* the phosphorylated active form of the enzyme is allosterically regulated by glucose. The earliest crystallographic information on glycogen phosphorylase came in 1974 with the publication of the T state phosphorylase *b* in the presence of the weak activator glucose-6-phosphate (Johnson *et al.*, 1974), this was quickly followed by high resolution data on the T state of phosphorylase *a* in the presence of the allosteric inhibitor, glucose. (Kavinsky *et al.*, 1976). Very recently the structure of the R state of phosphorylase *b* has been determined (Barford and Johnson, 1989). Comparison of this structure with the others already obtained gave information on the nature of the allosteric transition in glycogen phosphorylase.



The allosteric transition involves small changes in tertiary structure in the ligand binding sites and subunit interface regions and little change in the remainder of the subunit. These are coupled with large changes in the quaternary structure which involve rotation of the two subunits of the dimer with respect to each other. The change in quaternary structure directly effects the the allosteric effector site and the serine phosphate.

Glycogen phosphorylase is a complex enzyme in terms of its regulation and its variable quaternary structure, however, these recent studies are an excellent example of how allosteric effectors exert their affects at the intersubunit interface.

### 1.5.3. Lactate Dehydrogenase

Lactate dehydrogenase from *Bacillus stearothermophilus* has been extensively studied by site-directed mutagenesis. At cellular protein concentrations this enzyme is known to be a dimer with poor substrate affinity. These do not readily associate to form tetramers as this would bring together four positively charged pockets within repulsive range. In the presence of the allosteric effector, fructose 1,6 bisphosphate these charges are neutralised and the dimers can associate to form the high activity, high affinity tetramer. Site directed mutagenesis has been used to alter the charge in the substrate binding pocket, this does not affect the binding of the effector, nor does it alter the catalytic activity of the inactive dimer, but allows formation of the tetrameric form of the enzyme ( Clarke *et al*, 1989). This does not, however, mimic the activation induced by effector binding. The allosteric activation of the enzyme by fructose 1,6 bisphosphate involves a tertiary structural change as well as an alteration in the quaternary structure. (Matsuzawa *et al*, 1988)

#### 1.5.4. Phosphofructokinase

The enzyme phosphofructokinase catalyses the phosphorylation of fructose-6-phosphate to form fructose-1-6-bisphosphate. The enzyme is a tetramer of four identical subunits. Phosphofructokinase is allosterically activated by ADP and GDP and inhibited by phosphoenolpyruvate, the substrate of the pyruvate kinase reaction. The allosteric mechanism of the enzyme has been extensively studied, as crystals of both the R and the T states of the enzyme are available (Shirakihara and Evans, 1988, Schirmer and Evans, 1990). The allosteric transition again takes the form of a rotation of two halves of the tetramer relative to each other (Schirmer and Evans, 1989).

In the R state of phosphofructokinase, parallel  $\beta$  strands of neighbouring subunits are linked at the interface by hydrogen-bonded water molecules. In the T state these water molecules are expelled and direct hydrogen bonds are observed between the  $\beta$  sheets. The relative rotation of the subunits directly affects the catalytic site of the enzyme which lies near the subunit interface. There is an arrangement of the amino acid side chains within the active site of the enzyme on transition to the R and the T state such that the ionic environment switches from cationic to anionic repelling the substrate fructose-6-phosphate. ATP binding is also made less favourable after this transition.

Phosphofructokinase is also cooperative with respect to the substrate fructose-6-phosphate yet not with ATP. This can be explained by the fact that fructose-6-phosphate binding affects the intersubunit interface which is involved in the allosteric transition, while ATP binds within a subunit domain and is not sensed at this interface. The situation may be analagous to that observed in pyruvate kinase which is cooperative with respect to the substrate

phosphoenolpyruvate but not ADP.

The allosteric control of phosphofructokinase has also been studied by site directed mutagenesis (Lau and Fersht, 1987, Lau *et al.*, 1987). One very interesting result was obtained with the mutation E187A, a change from a glutamate to an alanine at position 187. This caused the allosteric inhibitor phosphoenolpyruvate to become an activator, with the phosphoenolpyruvate-bound R-state having a  $V_{max}$  only 40% of the wild type. Recent structural studies have confirmed that E187 is one of the side chains which is reoriented during the allosteric transition. In the R state this side chain binds  $Mg^{2+}$  and K213. The mutation E187A alters the effector binding site such that the allosteric equilibrium of the phosphoenolpyruvate complexed with the enzyme is shifted to the R state, and alters the tertiary structure of the R state such that the catalytic activity is reduced. Other mutations in and around the effector site back up the model of the allosteric transition derived from the crystallographic data.

#### 1.5.5. Aspartate Carbamoyltransferase

The enzyme from *Eschericia coli* has been extensively studied. The enzyme is composed of two catalytic trimers and three regulatory dimers. The holoenzyme is readily separated into a catalytic and a regulatory portion by treatment with mercurials or mild heat. When examined separately it can be seen that the holoenzyme and the catalytic segment of the enzyme differ kinetically in that the specific activity of the catalytic subunit is only 50% of that of the holoenzyme, and the substrate saturation curves for the catalytic portion of the enzyme are no longer sigmoid, indicating the loss of cooperativity (Kantrowitz and Lipscomb, 1990). Substrates are known to bind to the catalytic fragment while effectors have been shown to bind to the



regulatory portion of the enzyme (Gerhart and Schachman, 1965). Aspartate transcarbamoylase has been reported to be a model for the Monod concerted allosteric transition (Kantrowitz and Lipscomb, 1990), however these authors report that substrates and substrate analogues for the enzyme can induce the allosteric transition from the T to the R state. The alterations to the quaternary structure in the face of substrate binding has been studied by X-ray crystallography (Herve, *et al.* (1985)). The allosteric transition in aspartate transcarbamoylase has been likened to the functioning of a differential gear (Perutz, M.F. (1989)). The transition, which involves a rotation of one catalytic subunit to the other, has been quoted as being an example of a concerted structural change, in terms of the original Monod theory of allosteric control (Kantrowitz and Lipscomb, 1990) however aspartate transcarbamoylase may be a special case since this transition is brought about by substrates in this case.

#### 1.5.6. Allostery without conformational change.

The allosteric theories of Monod *et al.* and Koshland *et al.* cited at the beginning of this section both require a degree of conformational change to take place during the allosteric transition, whether this change be concerted or sequential. However a recent theory suggests that allostery may occur without conformational change (Cooper and Dryden, 1984). Here it is proposed that the allosteric effect may come about as a result of an entropic effect rather than a conformational one, or may occur in tandem with a conformational change. This paper also reports that cooperativity is also an entropic effect. In all reports of allosteric control of enzymes so far investigated, the allosteric transition is associated with conformation change. This is not the case for the

met and trp repressors. These proteins have no enzymic activity. They are DNA binding proteins which regulate expression of the enzymes required for methionine and tryptophan biosynthesis respectively by binding to specific operator sequences in the

genome of *E. coli*. Although the protein will bind to DNA alone, the affinity for its specific operator is increased by two orders of magnitude in the presence of the corepressor molecule S-adenosyl methionine (SAM). Corepressors can be regarded as somewhat analagous to allosteric effectors if one considers the repressor protein to be an 'enzyme' and the DNA as its substrate. The aporepressor crystal structure in the absence of SAM, the holorepressor in the presence of SAM, and the cocrystal structure in the presence of SAM and DNA have all been solved to a high resolution. (Rafferty *et al.* (1989), Somers *et al.* in press.). No significant change in tertiary or quaternary structure of the protein has been observed upon corepressor binding. The SAM analogue S-adenosyl homocysteine has been demonstrated to bind to the protein *in vitro*, but does not exert a corepressor effect. These two molecules are isosteric apart from a positive charge on the methyl group of SAM. It is implied that the 'allosteric' effect of the corepressor interaction hinges around the presence of this positive charge rather than any alteration of tertiary or quaternary structure.

### 1.6. Conclusion

How might this information be applied to the known structure of pyruvate kinase? Pyruvate kinase is a tetramer which, like phosphfructokinase and glycogen phosphorylase, can be viewed as a dimer of dimers, the 1,3 intersubunit interface being much less extensive than the 1,2 intersubunit interface. The 1,3 intersubunit interface in pyruvate kinase is strongly implicated in the

allosteric control of the enzyme from sequence comparisons of the allosterically regulated M2 isoenzyme in mammals with the non-allosterically regulated M1 isoenzyme. These two enzymes differ only in the sequence around the 1,3 intersubunit interface. Attempts to crystallise pyruvate kinase in the T state have so far been unsuccessful. It is tempting to speculate, in the light of the evidence from other proteins discussed above, that the allosteric transition in pyruvate kinase involves rotation about the 1,3 intersubunit interface, and that, in the M1 isoenzyme, this rotation is prevented by the amino acid sequence changes.

The rationale of this project was to study the allosteric transition of pyruvate kinase using the yeast enzyme as a model. The plan was to engineer site specific mutants of the yeast enzyme, mutated within the 1,3 intersubunit interface and express these in a *pyk<sup>-</sup>* background to permit their subsequent purification and characterisation.

## Chapter 2

### 2.1. MATERIALS

All chemicals were of highest grade available and purchased through Sigma, Poole, Dorset with the following exceptions, Phosphoenolpyruvate (PEP) and glass beads for yeast cell disruption from BDH. Growth media were supplied by Difco Labs., X-ray film and radiochemicals were supplied by Amersham International with the exception of  $\gamma^{32}\text{P}$ -dATP which was supplied in crude form from New England Nuclear. Rabbit muscle pyruvate kinase, used as protein gel standard was supplied lyophilised by Sigma, Lactate dehydrogenase was obtained from Boehringer Manneheim as a saturated ammonium sulphate slurry. All restriction enzymes were obtained from BRL, as was agarose for gel electrophoresis. All organic solvents were of the highest grade available and purchased from BDH either through the department of Biochemistry when ordered in bulk, or directly by our laboratory. Oligonucleotides were synthesised by the Oswell DNA synthesis service at the Department of chemistry, University of Edinburgh.

### 2.2. STRAINS AND PLASMIDS

#### 2.2.1. *E. coli* strains

HB101 *F*<sup>-</sup>, *hsdS20*, *recA13*, *ara14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl5*, *mtl1*, *supE44*

TG1 *K12*,  $\delta$  (*lac-pra* *supE*, *thi-1*, *hsdD5/F'traD36*, *proA+B+*, *lacIq*, *lacZ* $\delta$ M15)

BW313 *HfrKL16*, *Po/45*, *thi-1*, *dut-1*, *ung-1*, *supE44*.



### 2.2.2. *S. cerevisiae* strains

SF747 *mat- $\alpha$ , leu2, ura3, trp1, gal10*

$\delta$ pyk9 *mat- $\alpha$ , leu2, ura3, trp1, gal10, pyk1::ura3*

### 2.2.3. Vectors and Clones

pK19 (Pridmore R.D. (1987))  
 pUC18,19 (Yanisch-Peron C. et.al. (1985))  
 pJDB207 (Beggs J.D. (1981))  
 M13mpl8,19 (Yanisch-Peron C. et.al. (1985))  
 pAYE434 (fig11)  
 pMA91-pyk (fig11) (Mellor et.al. (1987))  
 M13-ab1 (fig12)  
 M13-ab2 (fig12)  
 pPYK20 (fig12)  
 pKpyk (fig12)  
 pPYK1E (fig12)  
 pPYK2E (fig12)  
 pPYK3HE (fig12)

The vector pJDB207, and clones M13-ab1, pPYK1E, pPYK2E, pPYK3HE and pAYE434 were a kind gift from Dr. A.J.P. Brown, (Dept. of Microbiology, University of Aberdeen.) The plasmid pMA91-pyk was generously provided by Dr. Jane Mellor (Dept. of Biochemistry University of Oxford.) The *E. coli* strain BW313 was donated by Dr. J.M. Miles (Department of Biochemistry, University of Glasgow.) pUC and M13 vectors were supplied by Gibco-BRL, Paisley, Scotland.

## **2.3. METHODS**

### 2.3.1. Growth of *E. coli* strains

(a) M9 minimal medium.

This medium was used for the growth and maintenance of the F' strains, TG1 and BW313 to select for pilus formation and thus susceptibility to M13

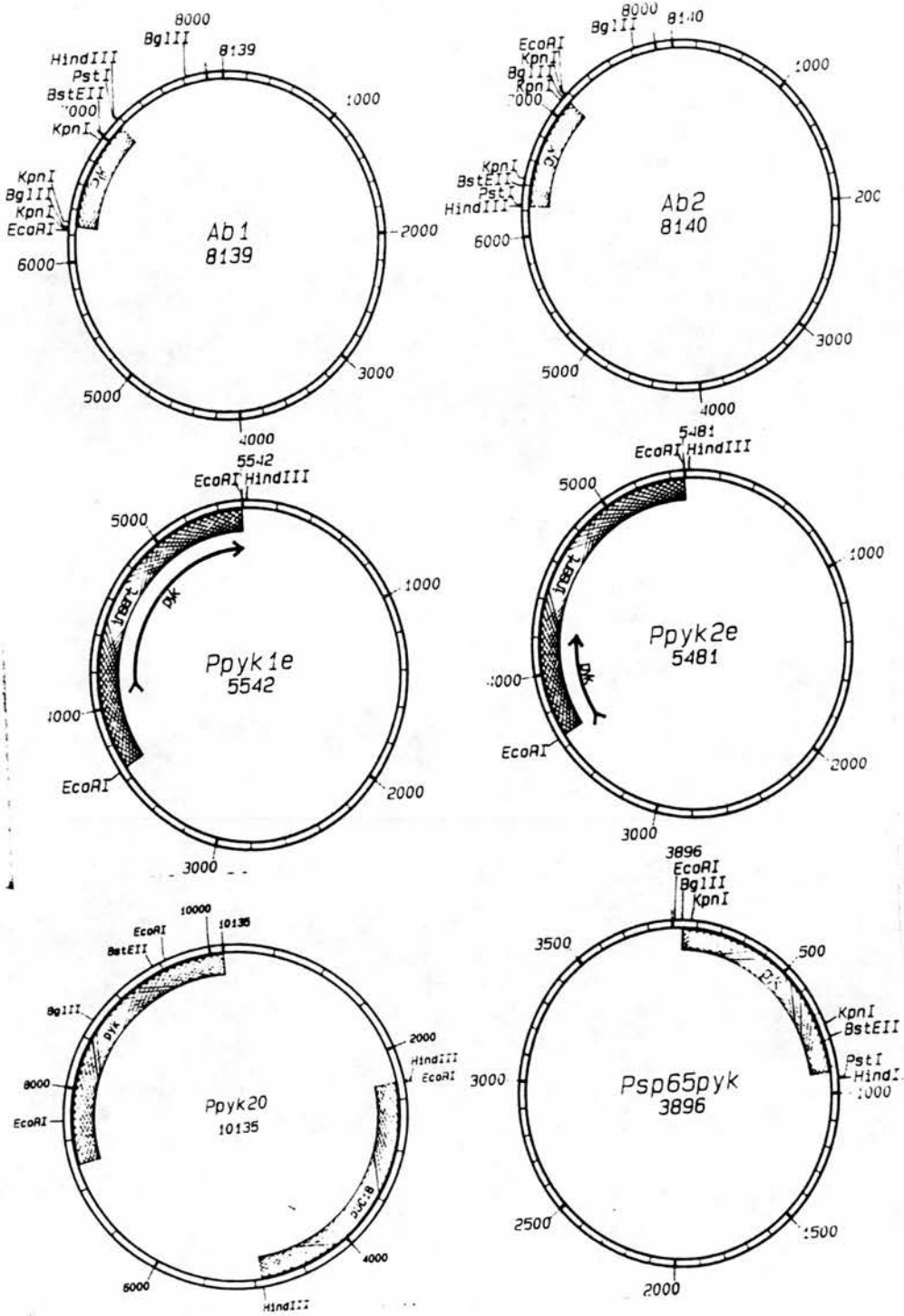


Figure 11; Plasmid maps of the *E. coli* plasmids employed throughout this project.

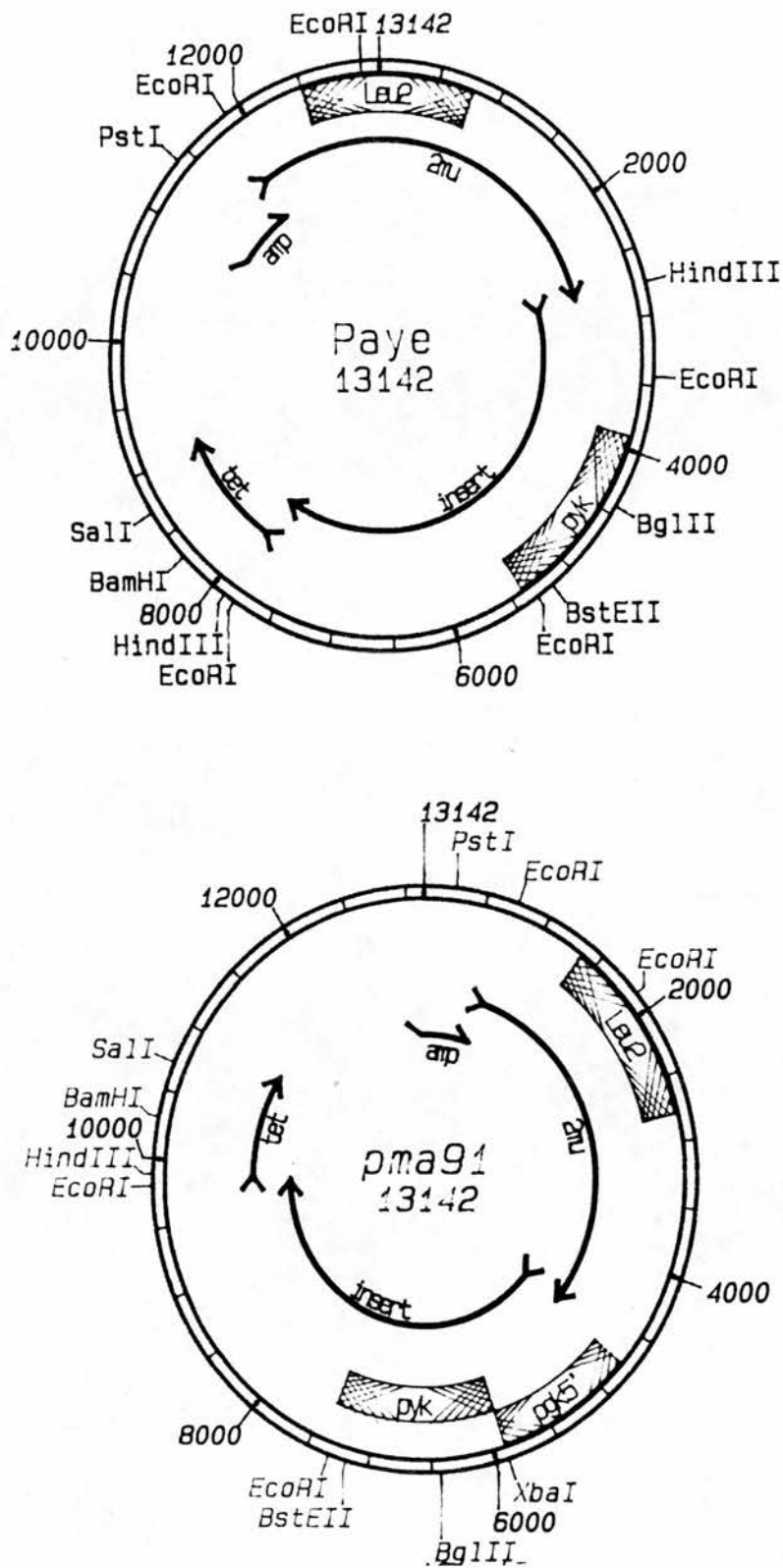


Figure 12: Plasmid maps of the shuttle vectors employed to overexpress pyruvate kinase.

infection.

#### M9 Minimal salts

60g/l  $\text{Na}_2\text{HPO}_4$   
 30g/l  $\text{KH}_2\text{PO}_4$   
 5g/l  $\text{NaCl}$   
 10g/l  $\text{NH}_4\text{Cl}$

This solution was autoclaved at  $15\text{lb/in}^2$  for 15 min then stored at  $4^\circ\text{C}$ .

#### M9 minimal agar.

15g of purified agar was dissolved in 860ml of distilled water by boiling, then autoclaved at  $15\text{lb/in}^2$  for 15 mins. The following ingredients were sterilised separately then added aseptically to the agar solution along with Thiamine HCl to a concentration of  $5\mu\text{g/ml}$  from a filter sterilised stock.

100ml M9 salts  
 20ml 20% glucose  
 10ml 0.1M  $\text{MgSO}_4$

When BW313 were grown on this medium a supplement of  $50\mu\text{g/ml}$  uridine was added to boost the intracellular dUTP pool.

(b) 2xTY medium other bacterial strains ie HB101 and all M13 infected strains were grown on 2xTY medium.

16g/l tryptone  
 10g/l yeast extract  
 5g/l  $\text{NaCl}$

This is autoclaved at  $15\text{lb/in}^2$  for 15 min To make a solid medium 15g/l purified agar was added prior to autoclaving. TY top agar was used in the growth of M13 phage, in this case agar was added to a concentration of 6g/l.

#### 2.3.2. Growth and maintenance of Plasmid strains

All plasmids, with the exception of those derived from pK19, had ampicillin resistance markers. These were maintained by growth in 2xTY supplemented with  $100\mu\text{g/ml}$  ampicillin (sodium salt). Those plasmids derived from pK19

which carries the kanamycin resistance marker were grown in 2xTY supplemented with 25 µg/ml kanamycin sulphate. All strains were stored as 15% glycerol stocks. These were made by growing a 1ml culture of the strain under selective conditions then spinning down the cells and resuspending in 250 µl 2xTY + 15% glycerol. Samples were then stored at -20°C.

### 2.3.3. GROWTH AND MAINTENANCE OF M13 STRAINS

M13 bearing strains were propagated in TG1 from minimal medium to ensure that the strain maintained the F plasmid. Phage bearing cells were grown in 2xTY medium for 4-5 hours to minimise recombination between the phage molecules. Recombinant phage strains were maintained as PEG precipitated phage molecules in sterile 2xTY broth at 4°C where they were stable for many months, or as DNA in an ethanol precipitate stored at -20°C indefinitely.

### 2.3.4. Preparation of plasmid DNA

Plasmid DNA was prepared on three scales depending upon the purpose for which it was required. The plasmid preparation methods are exactly as described in "Methods in Enzymology" vol. 152. Where CsCl purification was required to yield a pure preparation for DNA cloning, a large scale preparation usually from 500mls of culture was resuspended in 10ml of 1.1g/ml CsCl with 200 µg/ml ethidium bromide and centrifuged at 50,000 rpm for 36 hours in the Ti70 rotor of a Beckman L8 ultracentrifuge. DNA bands were visualized under long wave U.V. light then removed by puncturing the tube wall with a 1ml syringe and wide bore needle. Plasmid bands were extracted with water saturated butanol until no more pink colour could be seen in the upper phase. DNA was precipitated by the addition of 2 vol SDW, 1/10 vol 7.5M

$\text{NH}_4\text{CH}_3\text{COOH}$  and 2 vol ice cold isopropanol. This was then incubated at  $-20^\circ\text{C}$  for 60 min and spun 10,000 r.p.m. for 15 min to pellet the DNA. The pellet was washed carefully with 80% ethanol, dried under vacuum then resuspended in TE buffer to a concentration of approximately  $1\mu\text{g}/\mu\text{l}$ .

## 2.4. CUTTING AND CLONING OF DNA

### 2.4.1. Restriction Enzyme Cleavage

All restriction enzyme cleavages were performed in KGB buffer (McClelland 1987) as described in the paper. For those enzymes which did not perform well in this system, ie. BstEII, the buffer supplied by the manufacturer was used. All incubations were carried out at  $37^\circ\text{C}$  for at least 1 hour except in the case of BstEII which was incubated at  $65^\circ\text{C}$ . Digests were carried out in a total volume of  $20\mu\text{l}$  and 1–3 $\mu\text{g}$  DNA were digested at a time.

### 2.4.2. Purification of DNA fragments from agarose gels

DNA fragments were separated by agarose gel electrophoresis using TBE as the electrophoresis buffer.

#### TBE Buffer

Tris base	54.0g
Boric acid	27.5g
0.5M EDTA	20.0ml
per litre, pH 8.3	

After electrophoresis at 100V for 2–3 hours bands were visualized on a short wave U.V. transilluminator for the minimum length of time to avoid damage to the DNA. Bands were cut out of the gel using a sterile scalpel blade and placed in a sterile dialysis bag with 0.5 ml 1x TBE buffer. Both ends of the dialysis bag were securely sealed, the bag immersed in an electrophoresis tank

and subjected to 100V for 2-3 hours until all of the DNA was seen to leave the gel slice using long wave UV illumination. The direction of the current was then reversed for 2 min to remove the DNA from the wall of the dialysis bag. The dialysis bag was then opened and the buffer containing the DNA removed. The solution was phenol extracted once to remove all residual agarose which would inhibit subsequent ligations, then the DNA precipitated with 0.4 vol 5M  $\text{NH}_4\text{CH}_3\text{COOH}$  and 2 vol isopropanol, spun for 30 min in a microcentrifuge, washed with 80% ethanol and dried under vacuum. The DNA was then resuspended in 10 $\mu\text{l}$  SDW.

#### 2.4.3. Ligation of DNA fragments

Ligations were performed in a total volume of 20 $\mu\text{l}$ , the components of the reaction mixture were as follows;

DNA in TE or $\text{H}_2\text{O}$	13 $\mu\text{l}$
10x ligase buffer	2 $\mu\text{l}$
10mM ATP	2 $\mu\text{l}$
1mM DTT	2 $\mu\text{l}$
T4 DNA ligase	1 unit

care was taken to ensure that the ratio of insert to vector in any ligation was approximately 10:1. Ligations were incubated at 15°C overnight. ATP and DTT stocks were filter sterilised and stored at -20°C.

10x Ligase Buffer comprised; 200mM Tris-HCl (pH7.4), 100mM  $\text{MgCl}_2$ .

Ligation reactions were monitored by agarose gel electrophoresis wherever possible.

#### 2.4.4. Preparation of competent cells

Competent cells were prepared by a modification of the method of Mandel and Higa (1970). 1ml of an overnight culture of cells was used to seed a 100ml

flask of 2x TY broth. This flask was incubated at 37°C with vigorous shaking until the culture reached O.D.600 = 0.3, this usually took 1.5–2 hours. Cells were spun at 5000 r.p.m for 5 min then resuspended in 50ml 100mM CaCl<sub>2</sub> in the cold. From this point in the preparation every attempt was made to keep the cells as close to 4°C as possible. The cells were incubated at 4°C for 1 hour then spun again as before. The pellet was taken up in 2mls ice cold 100mM CaCl<sub>2</sub> and left overnight at 4°C to ensure a high level of competence. Competent cells could be kept this way for up to a week after which time they were no longer sufficiently efficient in the uptake of DNA to be of use.

#### 2.4.5. Transformation of competent cells

Competent cells were transformed as follows;

300µl of cells were pipetted into a standard test-tube and placed on ice. An aliquot of DNA was added in a volume no greater than 20µl, containing optimally 10–100ng of DNA, this was incubated on ice for 20 min to 1 hour with occasional gentle mixing to allow adhesion of the DNA to the cell surface. The cells were then heat shocked at 37°C for 5 min and replaced on ice. 1ml of sterile 2xTY broth was added aseptically to each tube and the mixture shaken at 37°C for 1 hour to allow expression of the antibiotic resistance. Cells were then plated onto appropriate selective media and grown statically at 37°C overnight. This same basic protocol was used for the transformation of M13 with the following modification; Cells were heat shocked as described previously and replaced on ice, 200µl of an overnight culture of TG1 cells were added to each tube along with 3mls of molten top agar pre-cooled to 45°C. This was mixed gently by rolling and poured onto the surface of a dry 2x TY agar plate then incubated overnight as before to allow plaque formation.



## 2.5. GROWTH AND MAINTENANCE OF YEAST STRAINS

### 2.5.1. Media

Strains of the yeast *Saccharomyces cerevisiae* were grown on the rich medium, YPD, which comprised.

10g/l yeast extract  
20g/l peptone  
20g/l dextrose

Where the yeast strain was harbouring a plasmid, a synthetic medium was required to complement the nutritional marker on the plasmid. This comprised;

1.7g/l yeast nitrogen base (w/o amino acids)  
5g/l  $(\text{NH}_4)_2\text{SO}_4$   
20g/l glucose  
(for the *pyk1::uraIII* disrupted strain dextrose is replaced by  
20g/l pyruvate)  
10ml of amino acid omission stock.

Amino acids are added to the medium after autoclaving at 15 lb/in<sup>2</sup> for 15 min. Stock solutions of the amino acids are made up with particular amino acids omitted i.e. for Leucine omission stock (100x concentration)

Adenine	5.0g/l
L-Arginine	2.4g/l
L-Aspartate	12.0g/l
L-Glutamate	12.0g/l
L-Histidine	2.4g/l
L-Lysine	3.6g/l
L-Methionine	2.4g/l
L-Phenylalanine	6.0g/l
L-Serine	45.0g/l
L-Threonine	24.0g/l
L-Tyrosine	3.6g/l
L-Valine	18.0g/l
Uracil	2.4g/l

L-Tryptophan was autoclaved separately at a 100x stock concentration of 0.2% w/v and stored in the dark at 4°C. Where leucine was required in the medium it was added at a concentration of 60µg/ml. For growth of uracil was omitted from the medium to ensure selection for the disrupted allele.

All liquid media were made into solid plates by the addition of 2% pure

agar. All yeast media were autoclaved at 15 lb/in<sup>2</sup> for 15 min prior to use.

### 2.5.2. Plotting a growth curve

Growth rate experiments were performed on all strains. This was done using synthetic media as described above. The strain *pyk1::uraIII* was grown on Yo + 2% pyruvate with leu, ura omission stock. Other strains were grown on Yo medium + 2% dextrose with omissions as required. 100ml cultures were inoculated with cells of the appropriate strain and grown with vigorous shaking at 30°C. 1ml samples of culture were taken at recorded times and the O.D.<sub>600</sub> recorded, this measure of the light scattering of the culture is proportional to the cell density. The results were plotted on a graph of cell density versus time to give a growth curve.

### 2.5.3. Determining optimal Pyruvate for growth

The optimal concentration of pyruvate for growth of the strain *pyk1::uraIII* was measured by setting up a range of tubes of Yo minimal medium with varying concentration of pyruvate from 0-4% w/v. The pyruvate was added aseptically from a filter sterilised 20% stock solution. The cells were grown at 30°C. with good aeration for 20-30 hours after which the O.D.<sub>600</sub> was measured on 1ml samples from each tube to give a measure of the cell density.

### **2.5.4. TRANSFORMATION OF YEAST CELLS**

Yeast cells were transformed by two methods, the Li CH<sub>3</sub>COOH pretreatment method of Ito (1987), and the sphaeroplast transformation method of Burgers (1988). Both of these methods work well. Although Li CH<sub>3</sub>COOH was less laborious, it was necessary to include an overnight preincubation in rich media to allow expression of the defective *leu2-d* allele present on both of

the shuttle vectors used before plating onto selective media. Putative transformants were tested for recovery of the transformed plasmid in an *E. coli* strain in a plasmid rescue experiment.

#### 2.5.5. Plasmid Rescue from yeast transformants

1.5mls of overnight yeast culture was harvested by centrifugation, the supernatant discarded and the process repeated two further times so that the pellet from 4.5ml of overnight culture was eventually harvested. Cells were resuspended in 800µl of BME buffer;

0.9M Sorbitol  
0.05M Na<sub>2</sub>PO<sub>4</sub>  
1µl/ml 2-mercaptoethanol]

25µl 10mg/ml lyticase was added and the tube mixed by inversion and incubated at 30°C for 20-40 min until more than 90% of cells had formed sphaeroplasts, as determined microscopically. Sphaeroplasts were harvested by centrifugation at 6500 rpm in a microcentrifuge for 30 sec, then resuspended in 100µl 1M sorbitol. Cells were lysed by the addition of 800µl of lysis buffer;

100mM Tris-HCl(pH 9.7)  
50mM EDTA(pH 8.5)  
0.5% SDS

and incubated at 70°C for 20 min, 200µl 5M KCH<sub>3</sub>COOH was added and the mixture left on ice for 45 min. Cell debris was pelleted by a 10 sec. spin in the microcentrifuge and the supernatant carefully decanted, 550µl of ice cold isopropanol was added and the mixture left at room temperature for 5 min. The DNA was pelleted by centrifugation for 15 min and the pellet washed with 70% v/v ethanol and dried under vacuum then resuspended in 20µl TE buffer. 10µl aliquots of this DNA could be used to transform *E. coli* cells as described previously.

### 2.5.6. GENE DISRUPTION

A gene disruption was performed on the yeast strain SF747. This allowed the insertion of a *ura3* marker into the *pyk1* locus of the genome. This ensured that the expression of mutant protein was not hindered by a background of wild type contamination.

The gene disruption method used was that of Lee (1984). A bacterial clone was synthesised from pPYK20 by linearising this plasmid with BglII and ligating in the yeast *ura3* gene which was obtained on a BamHI fragment from Dr. G. Reid, Dept. of Microbiology, University of Edinburgh. This construct was cut with HindIII to give an 8.9Kb fragment of DNA containing the entire *pyk* gene with its upstream and downstream flanking sequences interrupted by the *ura3* coding sequence. These linear DNA fragments were transformed into the yeast cells by alkaline cation pretreatment as described **previously**. The linear ends have a 1000fold increased recombination efficiency and thus encouraged chromosomal insertion of the *ura3* gene into the *pyk* locus on chromosome 1 of the yeast genome. Transformants were selected by growth on Yo medium + 3% glycerol, 3% ethanol without uracil to select for complementation of the *ura3* auxotrophy of the yeast strain pJDB207. These were then tested for the ability to grow on glucose. Those where the gene was positioned in the *pyk* locus could no longer undertake glycolysis and thus could no longer use glucose as the sole carbon source. The method is summarised in figure 15

Transformants took 14 days to appear on the glycerol/ethanol medium so attempts were made to find a more efficient growth system. The best medium seemed to be Yo + 2% pyruvate.

### 2.5.7. Isolation of Yeast Chromosomal DNA

In order to characterise the disrupted strain further, a Southern blot of yeast chromosomal DNA was prepared from disrupted and control strains, and probed with both *pyk1* and *ura3* to illustrate the chromosomal insertion. High molecular weight chromosomal DNA was extracted thus;

Cells from a 5ml culture grown to an  $A_{550}$  of 5-10 were harvested by centrifugation and resuspended in 500 $\mu$ l 1M sorbitol. 20 $\mu$ l of 10mg/ml lyticase was added and the cells incubated at 30°C for 1 hour after which time the sphaeroplasts were pelleted by spinning for 2 min at low speed in a microcentrifuge. Sphaeroplasts were lysed by the addition of 50 $\mu$ l 10% w/v SDS and the tube incubated at 65°C for 20 min. 200 $\mu$ l 5M  $KCH_3COOH$  was added to precipitate the cell debris and the tube incubated on ice for 30 min. The debris was pelleted by centrifugation for 5 min in a microcentrifuge at 13000 rpm. The supernatant was decanted into a 1.5 ml Eppendorf tube and absolute ethanol added to precipitate the DNA. DNA was pelleted in the microcentrifuge by spinning at high speed for 5 min. The pellet was vacuum dried and resuspended in 300 $\mu$ l TE buffer. 50 $\mu$ l of 10mg/ml RNaseA was added and the tube incubated for 1 hour at 37°C. 500 $\mu$ l of isopropanol was added to reprecipitate the DNA. DNA was pelleted as before and then washed with 80% ethanol and vacuum dried. This DNA is a suitable substrate for restriction endonucleases.

### **2.6. SOUTHERN BLOTTING**

Southern transfer was performed as a modification of the method devised by Southern (1975). DNA was cut with the restriction enzyme *HindIII* and run out on a 0.7% agarose gel in TBE buffer until the bromophenol blue dye front

reached the end of the agarose gel slab. The gel was stained with ethidium bromide and photographed. The DNA was denatured in the gel prior to Southern transfer by shaking for 30 min in a solution of 0.5M NaOH and 1.5M NaCl. The gel was then neutralised by shaking for 1 hour with 1M Tris-HCl pH8.0 and 1.5M NaCl with 3 changes of buffer. There should be enough solution in each of these cases to cover the gel completely during the shaking procedure. The gel was set up for Southern transfer as shown in the paper. Transfer was judged to be complete when 1 litre of solution had transferred through the gel. Hybond-N was used as the transfer matrix. This has the advantage of being less brittle than nitrocellulose and need not be baked in a vacuum oven to fix the DNA, indeed, DNA was fixed to the membrane by 2 min exposure to UV light. The hybridisation method is described in section (2.7.7.)

## 2.7. SITE DIRECTED MUTAGENESIS

Site Directed Mutagenesis was performed by the method of Kunkel (1985) with the following modifications:

The strain of *E. coli* BW313 was used as this has no amber suppressor and so is suitable for use with M13mp18 and 19. *E. coli* strain TG1 was used as the ung<sup>+</sup> control.

### 2.7.1. Growth of Phage

A single plaque of M13ab1 was removed from a plate and placed in an Eppendorf tube containing 1ml of TY broth. This was then vortexed thoroughly and heated to 60°C for 5 min to kill all *E. coli* cells. The tube was then spun for 2 min to pellet agar and cell debris. The supernatant was then used to inoculate a flask containing 100ml of TY broth + 0.25µg/ml uridine to which 5mls of a log phase culture of BW313 had been added. This was grown with

good aeration for 18 hours.

### 2.7.2. Preparation of Phage

The culture centrifuged at 500xG for 30 min, the supernatant collected, mixed with 0.4 vol. 20% PEG/2.5M NaCl and incubated at 4°C for 1 hour. Phage were collected by centrifugation at 500xG for 30 min. All of the PEG/NaCl mix was carefully removed with a drawn out Pasteur pipette then the pellet resuspended in 1ml TE in an eppendorf. This was then spun for 30 min at 13000 rpm in a microcentrifuge to remove all possible remaining cell debris then reprecipitated with PEG/NaCl as before. This final phage pellet was deemed sufficiently pure to continue with extraction of the phage DNA. A small sample of these pure phage were used to titrate ung<sup>+</sup> and ung<sup>-</sup> cell lines. If there was less than 10,000 fold drop in ung<sup>+</sup> survival the DNA was not used for mutagenesis.

### 2.7.3. Preparation of DNA

The phage pellet was resuspended in 500µl of TE then phenol extracted 3x using a phenol solution equilibrated to pH8.0 with 0.5M Tris-HCl. The top layer was then extracted 2X with diethyl ether. The lower phase from the final extraction was then extracted with 24:1 chloroform:isoamyl alcohol. The final extract was divided into two Eppendorf tubes and mixed with 625µl absolute ethanol and 25µl 3M Na CH<sub>3</sub>COOH to precipitate the DNA. DNA was pelleted by spinning 15 min in a microcentrifuge at 4°C. This pellet was then washed with 1ml of 80% ethanol and dried under vacuum until there was no residual alcohol. The DNA pellet was taken up in TE to a concentration of 1µg per ml as determined by A<sub>260</sub>. This preparation yields approximately 150 µg of DNA.

#### 2.7.4. 3'-phosphorylation of oligonucleotides.

2.5µl oligonucleotide (5 OD units/ml stock from Oswell)  
 3.0 µl 10x kinase buffer (1M Tris-HCl pH 8.0, 100mM MgCl<sub>2</sub>, 70mM dithiothreitol, 10mM ATP)  
 25µl SDW  
 2u T4 polynucleotide kinase

This mixture was incubated at 37°C to allow the end labelling reaction to occur then heated to 70°C to destroy the enzyme. The oligonucleotide was either used straight away or stored at -20°C prior to use.

#### 2.7.5. Mutagenesis Reaction

The phosphorylated oligonucleotide was annealed to the single stranded DNA template as follows:

1 µl of template DNA  
 1 µl phosphorylated oligonucleotide  
 1 µl 10X Klenow reaction buffer  
 7 µl SDW

Klenow reaction buffer comprised 100mM Tris-HCl pH8.0, 50mM MgCl<sub>2</sub>. This mixture was heated to 65°C for 5 min then allowed to cool slowly in a beaker of water until the temperature dropped below 25°C. 1µl Klenow DNA polymerase (10 units) and 1µl T4 DNA ligase (1 Weiss unit) were added and the tube incubated at 15°C for 18 hours. A 1µl sample was taken at this stage to assess the efficiency of the polymerisation reaction. Single stranded DNA runs distinct from double stranded on a 1% agarose gel. 1µl and 5µl samples were then transformed into competent TG1 cells and incubated O/N at 37°C. Samples of the resulting plaques were picked and grown in 1.5mls TY broth seeded with 10µl of a log phase culture of TG1. In initial experiments 10 plaques were picked at a time and subjected to T tracking (as described in section 2.8.5.) to assess the efficiency of the mutagenesis reaction. Once it was demonstrated that the efficiency was around 80% ie out of any 10 plaques chosen at least 8 would be mutant, then 4 plaques were selected and sequenced fully using the Sequenase kit. In all cases at least 3 plaques out of



the four selected were seen to be mutants. A flow diagram of the Kunkel mutagenesis method is shown in figure 10

The mutant K269R was difficult to screen by sequencing alone since the site of the mutation was greater than 200bp from the sequencing primer and the sequence change from CAGAAT to CAAGAT was difficult to detect even on long run sequencing gels, so this mutant was screened using a radioactive labelled oligonucleotide then later sequenced to verify the mutation.

#### 2.7.5. Labelling of the oligonucleotide

10X kinase buffer (as before)  
 3µl  $\gamma$ -<sup>32</sup>P dATP  
 1unit T4 polynucleotide kinase  
 1µl oligonucleotide (5 OD units/µl)  
 18µl SDW

incubated at 37°C for 1 hour.

#### 2.7.6. Preparation of Filter

DNA prepared from putative mutant plaques was arranged on a Hybond-N filter using a Schleicher and Schuell slotbotting apparatus. Filters were prepared using the manufacturer's instructions. 5µl of each DNA clone was applied to individual wells of the apparatus. DNA was fixed to the filter by irradiation with UV light for 5 min. Filters were stored dry, wrapped in clingfilm prior to hybridisation.

#### 2.7.7. Prehybridisation/Hybridisation

Hybridisations were performed as a modification of Jones and Singh (1981) the solution comprised:

6X SSC  
 500µg/ml Heparin  
 0.2% SDS  
 0.1% Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>

The filter was prehybridised in 10ml of this solution, in a sterile petri dish, at room temperature for 1-3 hours then, after addition of the labelled probe, was hybridised for a further 18 hours. The filter was then washed at sequentially increasing stringencies to differentially remove the probe from the negative controls and reveal positive clones.

## 2.8. DNA SEQUENCING

DNA sequencing was performed by the chain termination method of Sanger (1977) using both Klenow DNA polymerase and latterly Sequenase<sup>r</sup>.

### 2.8.1. KLENOW SEQUENCING

Klenow DNA sequencing was performed as described in the Amersham M13 'Cloning and Sequencing Handbook.

### 2.8.2. Sequencing reactions

1) 10X Klenow reaction buffer: 1M Tris-HCl, 500mM MgCl<sub>2</sub> 2)

dNTP/ddNTP solutions

dCTP/ddCTP: 0.50mM ddCTP, 0.16mM dGTP, 0.16mM dTTP, 0.008mM dCTP

dTTP/ddTTP: 0.25mM ddTTP, 0.16mM dCTP, 0.16mM dGTP, 0.008mM dTTP

dGTP/ddGTP: 0.50mM ddGTP, 0.16mM dCTP, 0.16mM dTTP, 0.008mM dGTP

dATP/ddATP: 0.50mM ddATP, 0.16mM dCTP, 0.16mM dGTP, 0.160mM dTTP

3) Chase solution: 0.50mM dATP, 0.50mM dCTP, 0.50mM dGTP, 0.500mM dTTP

4) Formamide dye mix: 0.3% (w/v) xylene cyanol FF, 0.3% (w/v) bromophenol blue  
20mM EDTA in SDW.)

### 2.8.2. Growth of phage

One plaque of an M13 clone was aseptically inoculated into 1.5ml of TY broth seeded with 10µl of a log phase culture of TG1 and grown at 37°C with good aeration for 5-6 hours.

### 2.8.3. Preparation of template

The bacterial culture was spun in a microcentrifuge for 5 min to remove all bacterial cells. Phage were precipitated from the supernatant by addition of 0.4vol. 20% w/v PEG/2.5M NaCl and incubated at room temperature for 15 min. The phage pellet was spun down for 10 min in a microcentrifuge and the supernatant removed. The pellet was then spun again to bring down all the residual PEG/NaCl and this was carefully removed from the pellet with a drawn out Pasteur pipette. The phage pellet was then resuspended in 50 $\mu$ l TE and phenol extracted to remove the protein coat, then chloroform extracted to remove both residual phenol and PEG. DNA was precipitated by the addition of 1/10 vol 3M Na CH<sub>3</sub>COOH and 2 vol. ice cold absolute ethanol. This was incubated at -20°C for 1 hour, spun for 30 min in a microcentrifuge then vacuum dried to remove all traces of ethanol. This pure DNA was then resuspended in 20 $\mu$ l of TE.

### 2.8.4. Annealing of primer to template

5 $\mu$ l of DNA template  
 1 $\mu$ l of 1/500 dilution of oligo  
 1 $\mu$ l of 10X Klenow reaction buffer  
 3 $\mu$ l SDW)  
 Incubated at 65°C for 1 hour .

### Polymerisation

1 $\mu$ l [ $\alpha^{35}$ S]-dATP and 1 unit Klenow DNA polymerase were added to the annealed primer/template mixture. 2.5 $\mu$ l of this was placed in each of 4 Eppendorf tubes labelled A,C,G and T and placed in a microcentrifuge, 2 $\mu$ l of each of the dNTP/ddNTP mixes were added to their respective Eppendorf tubes and spun briefly to mix. The polymerisation reaction was allowed to continue for 20 min at which time 2 $\mu$ l of chase mix was added to each tube. After a

further 15 min incubation the reaction was stopped by the addition of 2 $\mu$ l of formamide dye mix. The samples were then electrophoresed immediately or stored at -20°C until required.

#### 2.8.5. Selective Screening/T-Tracking

4 $\mu$ l sequencing primer  
6 $\mu$ l 10X Klenow reaction buffer  
12 $\mu$ l SDW

Add 2 $\mu$ l of this mix to each of 10 Eppendorfs along with 2 $\mu$ l DNA template and incubate at 60°C for 1 hour to anneal the primer and template. Meanwhile mix 16 $\mu$ l dTTP/ddTTP mix with 2 $\mu$ l  $\alpha^{35}$ S-dATP and 2 units Klenow DNA polymerase. Add 2 $\mu$ l of this to each of the 10 Eppendorfs and incubate 20 min at room temperature. Add 1 $\mu$ l of chase mix to each tube then incubate for a further 15 min before adding 1 $\mu$ l of formamide dye mix to stop the reaction.

#### 2.8.6. Gel Electrophoresis and Autoradiography

40 % w/v acrylamide stock.

38g acrylamide  
2g NN'-methylenebisacrylamide  
distilled water to 100mls

Add 5g amberlite resin, stir gently for 30 min then filter through sintered glass filter to remove the resin. Store in the dark at 4°C.

10X TBE buffer

108.0g Tris base  
55.0g boric acid  
9.3g Na<sub>2</sub>EDTA.2H<sub>2</sub>O)

Make up to 1 litre with distilled water. This gives a stock solution of pH8.3

Sequencing gel stock

76.8g urea  
24ml Acrylamide stock

8ml 10X TBE  
Distilled water to 160ml,  
Use straight away or store in the dark at 4°C.)

#### 2.8.7. The sequencing gel

The BRL S0 gel electrophoresis system was used. This gives a gel of 40x20x0.4cm. The plates were washed thoroughly then rinsed in acetone and air dried to remove all traces of detergent from the surfaces. Two spacers of 0.4mm thickness were laid along the edges of the plates. The gel plates were then sealed together using BRL gel sealing tape to create the gel sandwich. The gel mix was prepared in a beaker, 80mls of sequencing gel stock with 60µl TEMED and 60µl freshly prepared 10% ammonium persulphate. The gel mix was then carefully poured between the gel plates, holding the plates at an angle of 30° to the lab bench and tapping the top surface of the sandwich to remove any trapped air bubbles. Once all air bubbles were removed the gel sandwich was laid horizontally on the lab bench and two sharks tooth combs were inserted flat face down to give a smooth gel top. The gel was then allowed to set for at least 30 min after which time the sealing tape was removed and the gel clipped onto the running frame. Buffer (1X TBE running buffer) was added to both reservoirs and the set-up checked for leaks. The combs were then carefully removed and inverted to give the sample loading wells. Sequencing samples were boiled for 3 min to denature the double stranded DNA then stored on ice before loading onto the gel. The gel was run at 60W constant power during which time the bromophenol blue and xylene cyanol dye fronts separated. The final run length varied depending upon the information required. A short run to the bromophenol blue dye front gave sequence information from 3-4 bp 3' of the primer up to approx. 150 bp away. Allowing the gel to run to the xylene cyanol dye front allowed approximately

another 50-100 bp to be read from a single gel run.

Sequencing gels were fixed in 12% methanol/10% acetic acid (v/v) for 30 min to remove urea, then dried for 90 min at 80°C. Autoradiography was performed using Amersham Hyperfilm in GRI film cassettes with intensifying screens. The films were exposed for 18-24 hours at room temperature.

### 2.9.1. SEQUENASE SEQUENCING

The Sequenase sequencing reactions were carried out exactly as described in the manufacturers instruction leaflet.

### 2.9.2. Sequencing reaction

- 1) 5X Sequenase buffer; 200mM Tris-HCl pH 8.0, 100mM MgCl<sub>2</sub>, 250mM NaCl.
- 2) 5X labelling mix; 7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP
- 3) dNTP/ddNTP solutions.
  - ddG; 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl.
  - ddA; 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl.
  - ddT; 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl.
  - ddC; 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl.
- 4) Stop solution; 95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanolFF.)

### 2.9.3. Annealing primer to template

Phage DNA was prepared as described above.

Primer	1µl of 1/500 dilution of Oswel stock
Sequenase buffer	2µl
DNA template	7µl (approx 2µg)

Were placed in a capped Eppendorf and heated to 65°C for 2 min then allowed to cool slowly to room temperature in a beaker of water prewarmed to 65°C.

#### 2.9.4. Polymerisation

Sequenase enzyme was diluted 1/8 in ice cold TE just prior to setting up the reaction.

The following was added to the annealed primer/template mix;

0.1M DTT 10μl

1x labelling mix	1µl
------------------	-----

 $\alpha^{35}\text{S}$ -dATP 0.5  $\mu\text{l}$ 

diluted enzyme                      2μl

This was mixed and incubated at room temperature for 5-10 minutes. Four Eppendorfs were set up labelled A, T, C, and G and 2.5µl of the respective dNTP/ddNTP mix was added to each. These were prewarmed to 37°C for at least 1 min before the addition of 3.5µl of reaction mix. Incubation was continued at 37°C for at least 5 min although incubation of up to 30 min is reported to have no effect on the sequencing reaction. Reactions were stopped by the addition of 2µl of stop mix (deionised formamide, 0.1% xylene cyanol, 0.1% bromophenol blue).

### 2.9.5. Gel electrophoresis and autoradiography

This was performed exactly as described above. Longer runs are possible with the Sequenase kit by using the 5X labelling mix undiluted in the sequencing reaction and allowing the reactions to run for the maximum recommended time (30 min) then it is possible to read 300-500 bp away from the primer.

Problems were encountered with both of these methods when sequencing the "corrected" area of the pyruvate kinase sequence. These are thought to be caused by stacking of the GC base pairs since this is a particularly GC rich area. These problems were overcome by the use of 7-deaza dGTP in the Klenow sequencing mixes in place of, and at the same concentration as dGTP. This derivative of dGTP can form only two hydrogen bonds with dCTP and so eliminates stacking problems. Substitution of the dGTP mixes of the

Sequenase kit with dITP as detailed in the manufacturers instructions did not alleviate the base stacking difficulties.

## 2.10. Protein preparation

### Preparation of CibacronBlue-Sephrose

CibacronBlue 3GA was purchased from Sigma(Poole, Dorset) Sepharose 6B was purchased from Pharmacia(Uppsala, Sweden) and was supplied preswollen in 1 litre batches.

To couple the dye to the matrix 1g of the dye was added to 100mls of the Sepharose suspension and the mixture was brought to pH 11.0, then stirred gently at room temperature overnight to catalyse the binding of the dye to the gel. The gel was then washed extensively with distilled water until no more blue dye could be eluted and poured into a column 16 x 3 cm and equilibrated with 200 mls extraction buffer.

### 2.10.1. Protein extraction from yeast

#### (1) small scale

Small scale protein preparations were performed on yeast cultures to ascertain the degree of overexpression of pyruvate kinase. In these instances a 10mls yeast culture was harvested and concentrated into an Eppendorf tube. The cells were resuspended in 100 $\mu$ l of protein extraction buffer (50mM MES, 3mM MgSO<sub>4</sub>, 20% glycerol). 1 spatula tip of glass beads (40 mesh) was added and the sample vortexed for 2 min then cooled on ice for 2 min before spinning in a microfuge to remove all cell debris. The supernatant was removed and an equal volume of boiling mix added, the sample was then boiled for 5 min prior to SDS-PAGE.



## (2) Large (preparative) scale

This method was used to prepare large quantities of pyruvate kinase from yeast cells.

## Extraction buffer

50mM MES pH6.5  
3mM  $\text{MgSO}_4$   
12mM 2-mercaptoethanol  
20% glycerol

1 litre pMA91pyk in YEPD was grown to late log phase and cells harvested 1000xG 5 min 250ml aliquots. All cells were pooled and repelleted. 10g glass beads (40 mesh) were added, +1mg PMSF, +1mg Benzamidine. The cells were lysed with a motorised homogeniser for 20 min. Total cell lysis ascertained microscopically. The beads were washed 10X with 20ml samples of extraction buffer to remove all traces of the enzyme. The sample was spun 5000xG 30 min to remove insoluble debris, made 40% saturated with  $(\text{NH}_4)_2\text{SO}_4$ , stirred for four hours, then spun 10000xG for 30 min to pellet some of the contaminating proteins. The supernatant was then made 70% saturated with  $(\text{NH}_4)_2\text{SO}_4$ , stirred for four hours, then spun 10000xG 30 min. The pellet was resuspended in 5mls extraction buffer then desalted over a sephadex G-25 column 28.0 x 2.5 cm, pre-equilibrated with extraction buffer. The sample was recovered in a volume of 50-60 mls, so was concentrated using an amicon filter to 20 mls before loading onto the cibacronBlue column. The protein sample from the amicon was loaded onto the blue column and allowed to equilibrate for at least 1 hour to optimise protein/dye interaction, the column was then washed with 100 mls extraction buffer to remove all unbound proteins, then the pyruvate kinase was specifically eluted with the following buffer;

## Elution Buffer

50mM MES pH6.5  
12mM 2-mercaptoethanol  
3mM  $\text{MgSO}_4$   
1mM F-1,6-bisP

## 1mM PEP

The pyruvate kinase was eluted by allowing this buffer to flow through by gravity, which took approximately 2 hours. The pyruvate kinase containing peak was collected, pooled and concentrated using the amicon filter, to a volume of 10 mls, then again passed over the sephadex G-25 column to remove all residual phosphoenolpyruvate and fructose 1,6 bisphosphate. The protein was found to be most stable as an  $(\text{NH}_4)_2\text{SO}_4$  pellet, so all pyruvate kinase containing fractions were pooled and this was made 70% saturated with solid  $(\text{NH}_4)_2\text{SO}_4$ , stirred for 1 hour then spun 15000xG 30 min to pellet protein, this was then stored at room temperature as a pellet.

## 2.11. SDS-PAGE

The "Mighty Small" SE200 protein gel apparatus (Hoefer Scientific, California) was used throughout to run discontinuous SDS gels (Laemmli, 1970). The gel sandwich was assembled according to the manufacturer's instructions and placed vertically on a glass plate which had been overlayed with a solution of 1% agarose in 1X Tris-Glycine. This sealed the plates to prevent leakage of the polyacrylamide gel. A 9% SDS-PAGE gel was usually employed to demonstrate the purity of the pyruvate kinase. This was run at 20mA for 1 hour before staining.

## 30% acrylamide solution;

acrylamide	58.4g
N,N Methylene bis-acrylamide	1.6g
Distilled Water	200mls
Stored at 4°C in the dark.	

## Separating gel buffer (1.5M Tris-HCl pH8.8);

Tris base	36.3g
Distilled water	200mls
adjust to pH8.8 with HCl	

## Stacking gel buffer ( 0.5M Tris-HCl pH6.8);

Tris base	3.0g
Distilled water	200mls
adjust to pH6.8 with HCl	

## 10% SDS;

SDS	50g
Distilled water	500mls

## 2X Sample loading solution;

0.5M Tris pH6.8	2.5mls
10% SDS	4.0mls
glycerol	2.0mls
2-mercaptoethanol	1.0mls
Distilled water	to 10.0mls

## Running (Tank) buffer;

Tris base	12.0g
glycine	57.6g
10% SDS	40mls
Distilled water	to 4 litres

## Stain stock (1% coomassie-blue R-250);

Coomassie-Blue R250	2.0g
Distilled water	200ml

stir and filter through Whatman no.1 filter paper

## Stain solution(0.125% w/v Coomassie blue, 50% v/v methanol, 10% v/v acetic acid)

1% Coomassie-blue R-250	62.5mls
Methanol	250.0mls
Acetic acid	50.0mls
Distilled water	to 500.0mls

## Destain solution (50% methanol, 10% acetic acid);

Methanol	500mls
Acetic acid	100mls
Distilled water	to 1 litre

### 2.11.2. Sample preparation

Protein samples were diluted with an equal volume of sample loading buffer and boiled for 5 min prior to loading the gel. The gel was run at a constant current of 20mA until the dye front reached the bottom of the gel. Gels were stained by soaking for 30 min in stain solution, then destained by shaking in destain at 50°C in a shaking water bath, with frequent renewal of the solution until the gel background was colourless.

9% SDS-PAGE gels were used, these were made up as follows.

#### Separating gel(9%);

2.75ml 30% acrylamide stock  
2.4ml separating gel buffer  
3.7ml Distilled water  
50µl 10% APS  
50µl TEMED

#### Stacking gel(9%);

0.67ml 30% acrylamide stock  
1.25ml stacking gel buffer  
3.00ml distilled water  
50µl 10% APS  
5µl TEMED

### 2.11.3. Electrophoresis

The gel was run at 100V until the coomassie blue dye front reached the bottom of the gel. The gel was then removed from the running apparatus and the plates prized apart using a spatula. The gel was immersed in stain solution for 1 hour then destained in several changes of destain solution until the protein bands were clearly defined and the background colourless.

### **2.11.4. PYRUVATE KINASE ASSAY**

Pyruvate kinase was assayed by a modification of the method of Bucher and Pfeleiderer (1955). Assays were performed in a 1cm path length quartz cuvette in a total volume of 1ml. The cuvette contained 6 µmol PEP, 6 µmol ADP, 0.25 µmol NADH, one unit of lactate dehydrogenase, 15 µmol MgSO<sub>4</sub>, 100µmol KCl and 50 µmol MES pH 6.2. The reaction was started by the addition of pyruvate kinase and the progress monitored as the change in absorbance at 340nm.

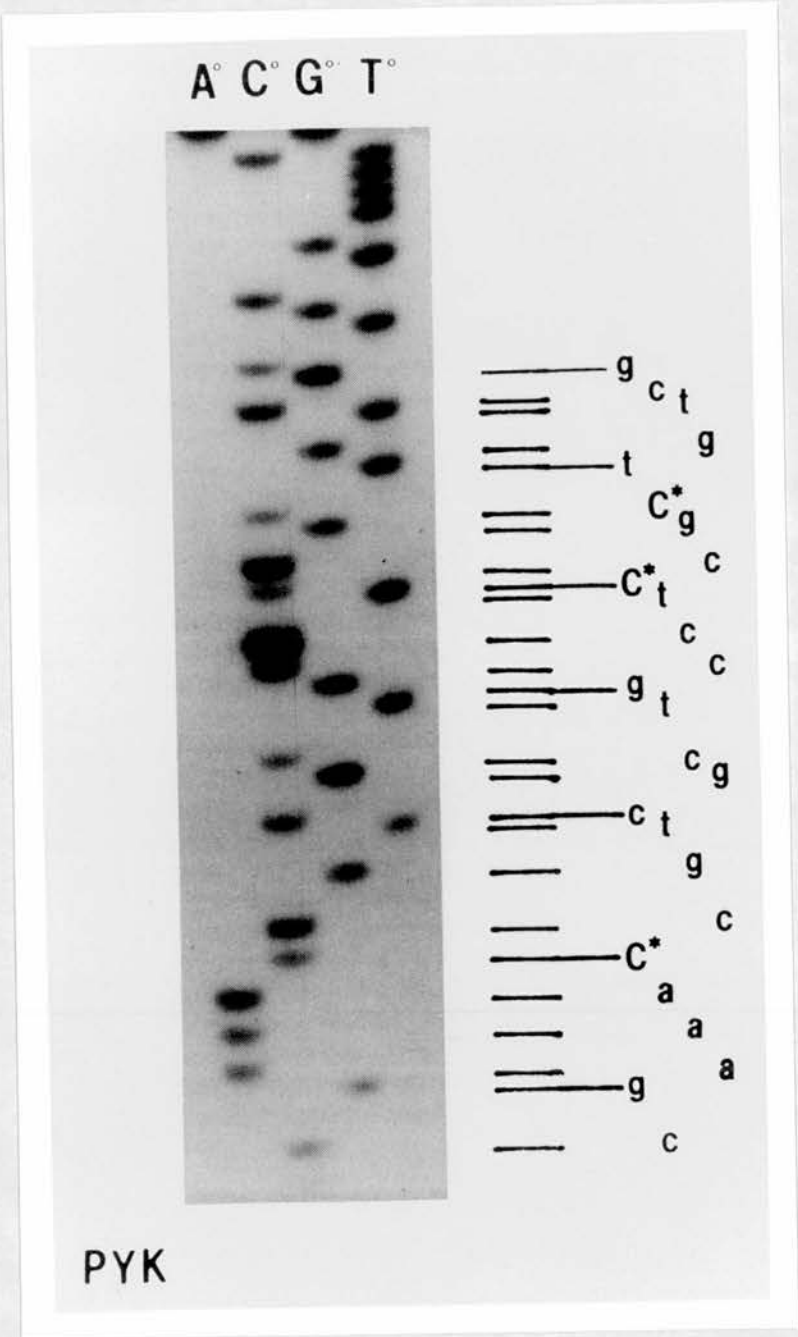
## chapter 3

### 3.1. SEQUENCING OF THE PYRUVATE KINASE GENE (*PYK 1*)

from *Saccharomyces cerevisiae*

#### 3.1.1. Background

The pyruvate kinase gene (*pyk1* from *Saccharomyces cerevisiae* had already been sequenced prior to the outset of this project (Burke *et al.*, 1983). This sequence was used to design specific oligonucleotides to direct mutations within the C $\alpha$ 1 helix of the intersubunit binding site. For these initial experiments the Amersham site-directed mutagenesis kit was used. Repeated rounds of mutagenesis failed to produce the desired mutations even though the controls, which are an integral part of the mutagenesis kit, indicated that the reactions were proceeding efficiently. When the mutagenic oligonucleotide was then tested as a sequencing primer it was discovered that it did not prime from the predicted point within the *pyk 1* gene, but rather from a point within the M13 genome. It was then assumed that this oligonucleotide had been directing a non-lethal mutation within the M13 genome. Other oligonucleotides produced no mutants and did not prime a DNA sequencing reaction. This region of the gene was sequenced and discovered to be different from that of the original published sequence of Burke *et al.* Dr. A.J.P. Brown, and his research group at the Department of Genetics, University of Glasgow, was also making site specific mutants of the *pyk 1* gene, using this as a model for translational pausing and protein folding. The central tenet of this theory requires a string of non-preferred codons within the coding sequence of a gene. Such a string is postulated to induce a pause in translation allowing the N-terminal part of the protein to fold before the C-terminal part is synthesised



ACC	ACC	GAA	ACC	GTC	GCT	GCC	TCC	GCT	GTC	GCT	GCT	GTT	
T	T	E	T	V	A	A	S	A	V	A	A	V	NEW
			T	S	L	P	R	-					OLD

Figure 13; The region of the pyruvate kinase gene found to be incorrect, the corrected sequence and the effect upon the protein sequence.

(Brown, 1987). The *pyk 1* gene was a strong candidate for translational pausing since it had 5 non-preferred codons in a row. Co-incidentally these codons were in the middle of the region of DNA which encodes the C $\alpha$ 1 helix. Attempts to mutate this region by Dr. Brown's group also met with failure. This region was sequenced by Dr. Brown's group who also observed a deviation from the original published DNA sequence. The sequence produced from both our laboratory and that of Dr. Brown had three extra 'C' residues. These were not arranged sequentially, so had the effect of putting the protein out of reading-frame for 4 residues and creating an extra codon (fig 13). The new sequence no longer contained any non-preferred codons making *pyk 1* a poor model for the translational pausing hypothesis.

### 3.1.2. The sequencing strategy

An essential requirement for oligonucleotide design in a mutagenesis project is a good DNA sequence. It was therefore decided to resequence the entire *pyk 1* gene. The sequencing strategy is summarised in fig 14. The region where the sequence discrepancy was found to occur was sequenced on both strands of the DNA, using an oligonucleotide primer which primed 15bp 5' to the first extra C residue in clone m13ab1 and by extending the reaction with universal primer on the reverse clone m13ab2. The N-terminal part of the coding sequence was cloned into m13 by excising the *eco*RI fragment from pPYK2E (fig 10) and cloning it into m13mp19, the resulting clone could then be sequenced using universal primer. No other sequencing discrepancies were observed in any other part of the gene.

The region where the sequencing discrepancy was found to occur is very 'GC' rich and this encouraged compressions making the sequencing gels very difficult to interpret. This common sequencing artefact was observed when



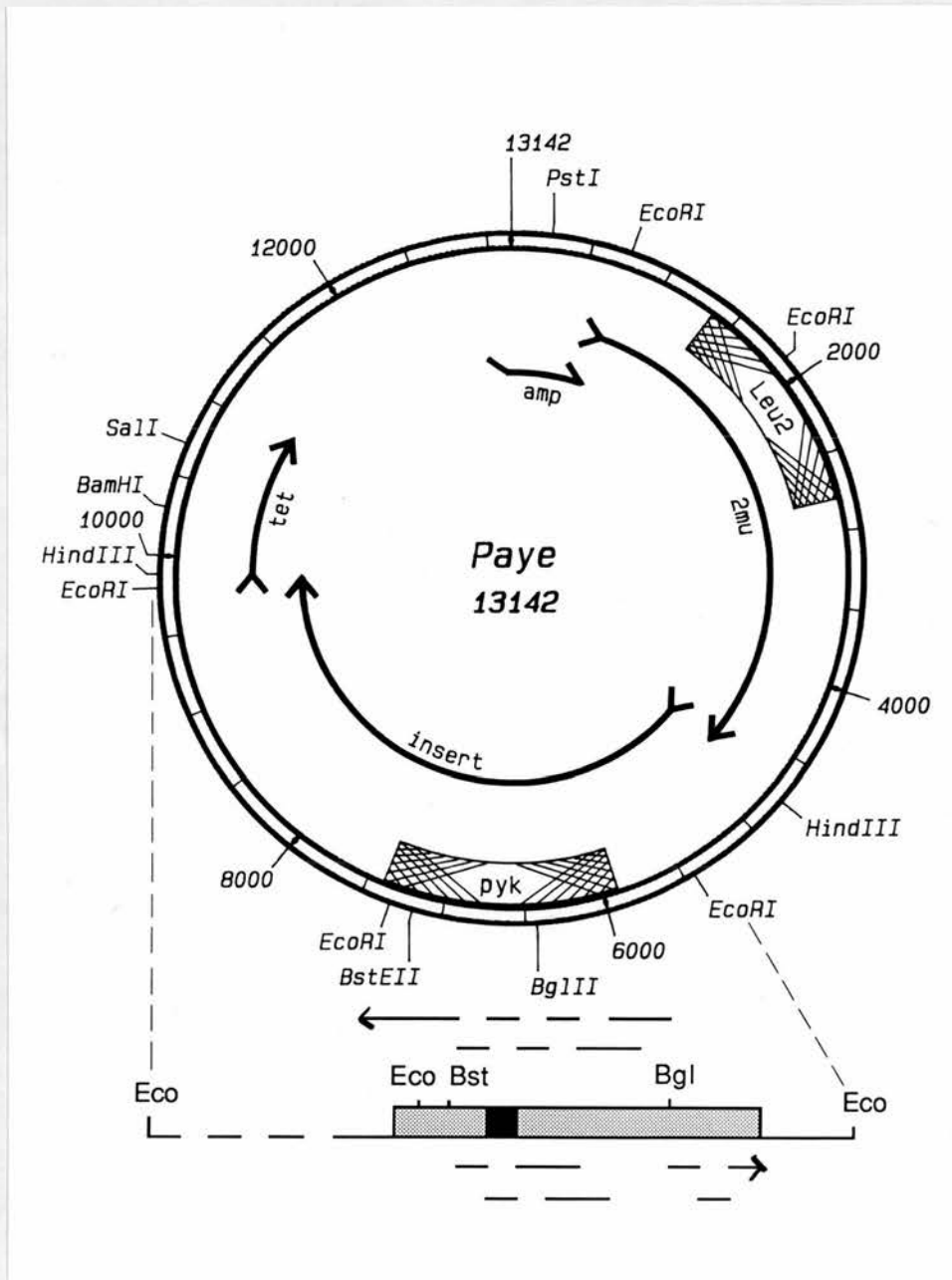


Figure 14; The sequencing strategy employed to resequence the *pyk1* gene.



both Klenow DNA polymerase and Sequenase were used to direct the sequencing reactions. Replacement of dGTP with dITP in the sequencing mixes did not alleviate this problem. The problem was eventually solved, however, by substituting dGTP with the synthetic nucleotide, 7-deazadGTP. Recent developments in DNA sequencing technology have developed the enzymes BST polymerase and TAQ polymerase. These enzymes can polymerise the DNA with high fidelity at temperatures up to 70°C, such that compression problems which result from strong secondary structures within the single stranded DNA are alleviated. The revised DNA sequence has been published, see McNally *et al.* 1989 in the appendix to this thesis. The sequence has been submitted to EMBL and has been given the accession number x14400.

## chapter 4

### 4.1. GENETIC MANIPULATION OF THE HOST YEAST STRAIN

#### 4.1.1 Gene disruption of the *pyk1* gene from *Saccharomyces cerevisiae*

When planning to overexpress any mutant yeast proteins in a homologous background it is desirable to utilise a host strain which is phenotypically mutant in the allele under investigation. Whenever possible it is advisable to utilise a strain which is totally deleted for the allele in question, such strains are created in the laboratory if not already available. In the case of the *pyk 1* gene this was not feasible for reasons that will be discussed at the end of this chapter. Instead a phenotypic mutant of *pyk1* was created by a process of gene disruption. This simply involved cloning a *uraIII* fragment into the central BglII site within the gene as described in figure 15. The resultant plasmid was then linearised with HincIII, the linear ends promoting recombination within the genome. The strain SF747 was used as the host in these experiments, the genotype of this strain can be found in the Materials and Methods section of this thesis.

Transformants from the gene disruption experiment took 12 days to grow. In total 14 transformants were obtained by selection on yeast minimal medium (Yo) lacking uracil with 3% glycerol and 3% ethanol as the carbon sources. The phenotypes of the disrupted strains are such that they can no longer utilise glucose as a carbon source as glycolysis is blocked. These cells accumulate a large quantity of phosphoenolpyruvate and die, however small carbon compounds which can enter the cell serve as alternatives. The lack of uracil in the medium selects for integrants within the *pyk1* locus. The transformants were tested for growth on glucose to test for functional *pyk*. Only five out of the 14 transformants obtained grew on glucose. The fact that

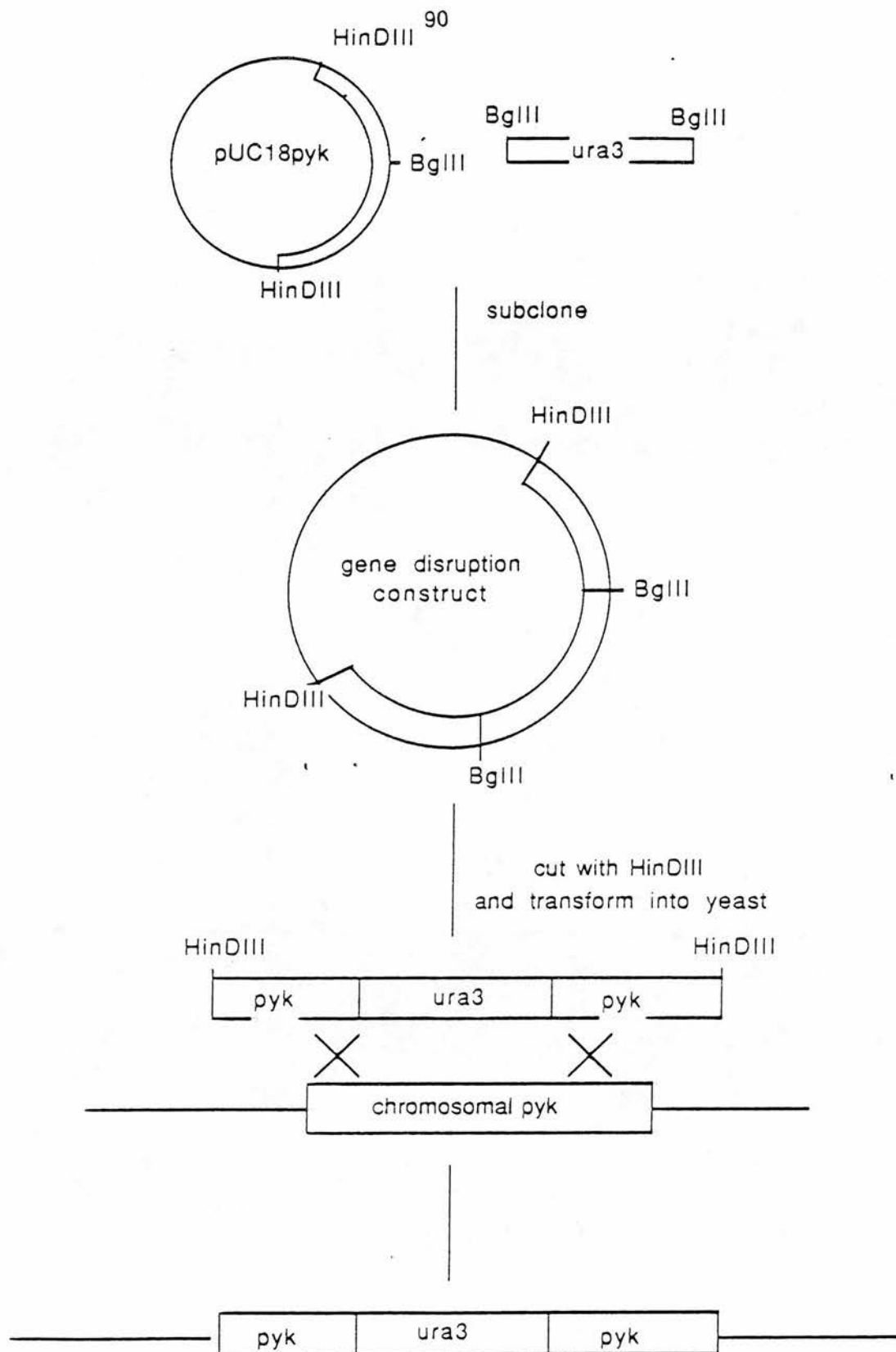


Figure 15: The mechanism of gene disruption.

these were all *ura*<sup>+</sup> suggests that the *uraIII* gene has recombined at another site on the chromosome, possibly the *uraIII* locus itself.

#### 4.1.2. Verification of potential disruptants.

Chromosomal DNA was prepared from the nine clones which looked promising from the nutritional selection tests. The DNA was cut with the restriction enzyme *HinDIII* and electrophoresed on a 1% agarose gel. The agarose gel was then Southern blotted and probed with the wild type pyruvate kinase gene in the plasmid pPYK20. The Southern is shown in figure 16. Two bands are apparent on this blot, the lower band can be attributed to the *pyk 1* gene and the upper band, either to an incomplete digest or some  $\beta$ galactosidase homologue.

#### 4.1.3. Optimising cell growth

Growth of the cells on glycerol and ethanol was very slow, colonies taking around five days to form on solid media. Ethanol is highly volatile and over prolonged incubation at 30° may evaporate from the medium. Glycerol is notoriously difficult to handle with any degree of accuracy. This prompted the investigation of a better growth medium.

Pyruvate ethyl ester was tested as a carbon source, the rationale being that this would enter the cell readily, being uncharged, and be broken down by non-specific esterases within the cell to yield pyruvate and acetate which could then serve as carbon sources to the cell. This compound was tested at concentration of 0.1–4.0%, but did not sustain cell growth. This must be because the ester is not entering the cell since both pyruvate and acetate alone are reported to support cell growth.

Pyruvic acid was tested for its efficiency as sole carbon source.

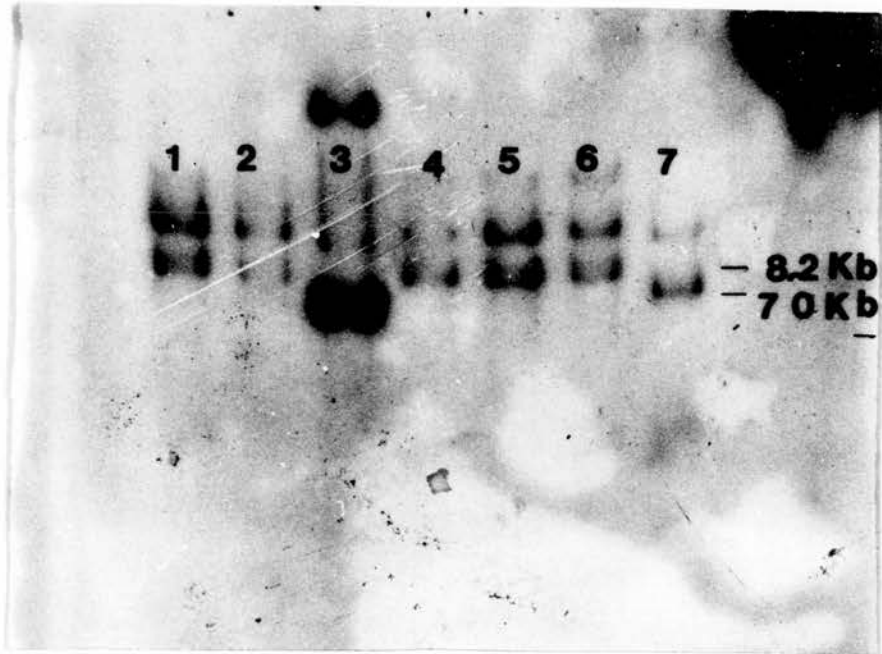


Figure 16: Southern blot of *HinDIII* DNA extracted from yeast clones obtained from gene disruption proed with a fragment of the *pyk1* gene. The wild type control in lane 7 illustrates that the *pyk1* gene is found on a 7Kb fragment. Lanes 1 - 6 demonstrate that in all but one case, the *pyk*-phenotype is associated with the insertion of the 1.2Kb *ura3* fragment resulting in an 8.2Kb product. The clone in lane 3 appears to have undergone a deletion which resulted in the *pyk*-phenotype.

Concentrations from 0.1% to 4% were tested to ascertain which permitted most extensive growth of the yeast strain. 2% pyruvate was seen to be the most effective growth substrate, higher concentrations eventually became inhibitory. There is no specific carrier for pyruvate in the yeast cell membrane, however pyruvate can enter the cell via the lactate/proton symporter in the membrane (Cassio *et al.*, 1987) Pyruvic acid was made up as a fresh 20% stock in distilled water then filter sterilised immediately prior to use. Old pyruvate would not serve as an effective carbon source, nor would pyruvate that had been autoclaved.

1

#### 4.1.4. growth of disrupted strain

Growth of the disrupted yeast strain was compared with that of the parent strain on synthetic medium + 2% pyruvate, both strains grew at a very similar rate, reaching the same cell density in stationary phase. This is further evidence to suggest that the only difference between the disrupted and non-disrupted strains is the loss of ability to grow on glucose. This strain was deemed suitable to use as a phenotypically *pyk*- background in which to overexpress mutant *pyk* genes.

#### 4.2. ADVANTAGES OF DELETION OVER DISRUPTION

Gene disruption is not the best technique to use for the purposes of this project, ie. overexpression of mutant proteins in a homologous background. Gene deletion is by far the method of choice. This is because, in the case of the gene disruption there is a small chance that recombination may occur between the mutant gene on a multicopy plasmid and the wild type gene which is present, although disrupted, in the genome. This could result in a wildtype gene on the multicopy plasmid which could quickly overgrow a cell population

if the mutant gene was in some way compromised compared to the wild type.

Attempts to create a strain of yeast deleted in the *pyk 1* locus failed to produce any transformants. It was initially assumed that this was due to a cloning aberration at one of the early stages in the process, however, this approach has also been attempted in another laboratory (Dr. A.J.P. Brown personal communication) with no success. This absolute requirement for *pyk 1* would not seem to be at the protein level as the disrupted strain of yeast has no active pyruvate kinase yet is viable. It is postulated that it may be the 5' end of either the DNA or the mRNA which is important for cell survival. This phenomenon is currently under study in Dr. Brown's laboratory.

Disruptants were further characterised by determining whether any active pyruvate kinase was produced by the disrupted strains. A 10ml culture was grown under appropriate selection, the cells harvested and lysed as described in section 2.10.1. The debris removed by centrifugation and the supernatant assayed for the presence of active pyruvate kinase by the method described in 2.11.4. No active enzyme was ever found.

## chapter 5

### 5.1. THE OVEREXPRESSION OF PYRUVATE KINASE

#### 5.1.1. Attempts to overexpress the protein under the control of its own promoter

Pyruvate kinase was initially overexpressed from the *Saccharomyces cerevisiae*/*E.coli* shuttle vector pJDB207 (Beggs *et al.*, 1983), the plasmid pAYE434 comprised the pJDB207 shuttle vector with a 7kb HindIII fragment of *Saccharomyces cerevisiae* DNA which was known to bear the *pyk1* gene. This plasmid is shown in fig 12.

The vector pJDB207 is a high copy number vector containing the *leu2d* allele. This allele is known to increase plasmid copy number. The precise mechanism is not known but it is speculated that, since this allele is mutant, only those clones where the vector has increased copy number can successfully complement the *leu<sup>-</sup>* phenotype of the host strain. Overexpression of proteins in this vector is by virtue of the high copy number as the vector contains no promoter sequences. Thus the overexpression of pyruvate kinase is directed by its own upstream sequences.

The strain SF747(*pyk1::ura11*) was transformed with the plasmid pAYE434, by sphaeroplast transformation as described in the 'methods' section of this thesis. Transformants were initially selected on omission medium with pyruvate as the carbon source and lacking leucine and uracil. All transformants were then tested on omission medium with glucose as the carbon source, lacking leucine and uracil. In no case were any transformants found to grow on pyruvate but not glucose, indicating the presence of a functional *pyk1* gene. The repeated selection for uracil and leucine ensured that the chromosomal copy of the *pyk1* gene remained disrupted and therefore non- functional, and that the clones



retained the plasmid. Additional controls to ensure that the transformants were genuine included plasmid rescue and restriction mapping. Initially, in all cases the plasmid pAYE434 was rescued from the yeast strain intact as far as could be determined by restriction mapping.

Protein was purified from both SF747 and SF747 + pAYE434 to determine the level of overexpression of pyruvate kinase. The level in the transformed strain was determined to be between 12 and 20 fold greater than in the SF747 strain. Protein gels of the cell extracts were difficult to interpret, as the levels of overexpression were not sufficiently high to identify, unambiguously, an increased level of pyruvate kinase by coomassie blue staining.

The transformed cells were stored at 4°C for a number of months before being used to seed cultures for large scale protein production. The protein preparation yielded only small quantities of pyruvate kinase, similar to the amounts expected for wild type strains. Repeated retransformations yielded positive transformants, but the levels of protein overexpression always decreases on large scale culture or on storage at 4°C. Other workers in this field also reported this problem, that when pyruvate kinase is overexpressed from its own promoter, the levels of protein overproduction decreased with time and culture (Dr. A.J.P. Brown, Mr. T.A. Murcott, personal communications).

#### 5.1.2.Overexpression under the control of the *pgk* promoter.

Another pyruvate kinase clone was obtained, this one having the entire pyruvate kinase coding sequence under the control of the *pgk* promoter. This plasmid, pMA91pyk, derived from the shuttle vector pMA91 is illustrated in fig 12.

The *pgk* promoter is one of the strongest known in *S.cerevisiae* permitting

expression of PGK to up to 80% of the total cell protein when present on a multicopy vector ( Mellor, *et al*, 1986). Transformation of this plasmid into the strain SF747 permitted massive overexpression of pyruvate kinase, to around 30% of the total cell protein, several hundred fold higher than wild type levels (fig 17) . This strain served as an excellent source of wild type pyruvate kinase protein in this project. This strain of yeast, SF747 + pMA91pyk, was tested for growth compared to the non-transformed strain SF747. The transformed strain demonstrated no reduction in growth rate as compared with non-transformed strain, clearly demonstrating that the overexpression of pyruvate kinase protein is in no way detrimental to cell growth. It may therefor be deduced that it is the 5' end of the *pyk1* which is responsible for the reduction in expression observed when SF747 is transformed with pAYE434. Work is currently underway to establish the nature of this decreased expression.

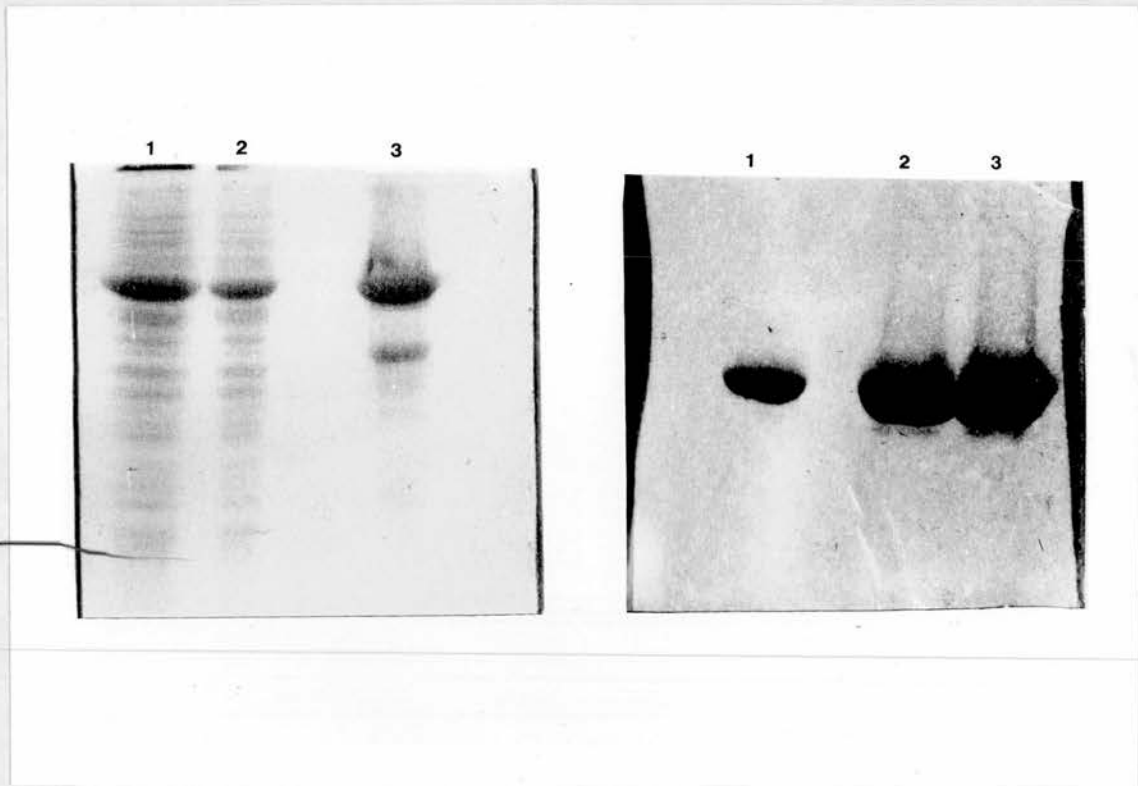


Figure 17; The left hand side illustrates the level of overexpression of pyruvate kinase from the shuttle vector pMA91. Small scale extracts were prepared as described in the materials and methods section (2.10.1.). The marker on the right hand side is cat muscle pyruvate kinase, supplied by Sigma. The right hand side shows a sample of pyruvate kinase purified by cibacronblue chromatography. The first two lanes represent 100 $\mu$ g and 500 $\mu$ g of protein, the third lane shows 500 $\mu$ g of the same preparation after storage had reduced its activity by 50%. There is no evidence of proteolytic degradation.

## chapter 6

### 6.1. SITE-DIRECTED MUTAGENESIS

#### 6.1.1. Background

The main purpose of this research project was to probe the allosteric control of the enzyme pyruvate kinase using site-directed mutagenesis techniques. The plan was to synthesize mutants of the enzyme within the 1,3 intersubunit interface: more specifically, within the C $\alpha$ 1 and C $\alpha$ 2 helices.

#### 6.1.2. Initial attempts at Mutagenesis

Oligonucleotides to direct these mutations were originally designed using the original published sequence of the pyruvate kinase gene of Burke *et al.* (1983). These initial attempts at mutagenesis were unsuccessful; of three 18mer oligonucleotides purchased to direct mutations within this area, only one would direct a mutation. Sequencing of a large number of putative mutant plaques obtained from a round of mutagenesis using the Amersham M13 mutagenesis kit did not reveal the expected mutation, even though the reactions appeared to proceed efficiently as judged by the integral controls within the kit. The oligonucleotides were used as sequencing primers using M13ab1 as the template. The two oligonucleotides which were previously shown not to direct a mutagenesis reaction would not function as sequencing primers. This suggests that the mutagenesis was failing due to inefficient hybridisation of the mutagenic oligonucleotide to the template DNA, the first step in any mutagenesis reaction. The oligonucleotide which did direct a mutation also functioned as a primer for de novo DNA synthesis. However, the oligonucleotide bound within the M13 vector sequence and not at the expected position within the pyruvate kinase gene. This would explain the fact that although a large number of putative mutant plaques were screened, the

expected mutation was not detected. It would now appear that a mutation was directed by this oligonucleotide but was not detected by screening as it was located within the M13 vector sequence.

#### 6.1.3. Mutagenesis within the active site of Pyruvate kinase

As mutagenesis within the region of the 1,3 intersubunit interface of the protein was hampered by the errors within the original published sequence, attention was focussed on the active site of the enzyme in an attempt to determine the role of the putative catalytic lysine residue 269.

Two oligonucleotides were designed to change lysine 269 to arginine and histidine. These oligonucleotides were 18 base pairs long. The sequences of the oligonucleotides used are illustrated below. The rationale behind these changes was to maintain the positive charge within the active site as this is probably required for enzymatic function, but to alter its location and study the effect on catalysis. In the case of the histidine which has a much lower pK than that of lysine and arginine and may not have been expected to be protonated at physiological pH, this change was postulated to alter the pH profile of the enzyme.

Mutagenesis was again carried out using the Amersham site-directed mutagenesis kit. However neither of these oligonucleotides would direct mutations within the active site of the enzyme. The sequence around this region of the active site was checked and found to be correct. The design of the oligonucleotides was also verified and no obvious errors were detected. In view of the previous lack of success with the mutagenesis reactions, the method of mutagenesis was changed from the Amersham kit, which is expensive and inflexible, to the Kunkel method of mutagenesis (Kunkel, 1985). This method is described in the methods section of this thesis. Attempts to

use these mutagenic oligonucleotides as sequencing primers again met with no success, this result could not be readily explained. Figure 18 shows the result of a dot-blot performed using one of the mutagenic oligonucleotides as a probe against M13ab1, the template for the mutagenesis reaction and M13ab2, the reverse of this clone. This result suggests that the oligonucleotides can bind to the template DNA, but cannot act as primers for de novo DNA synthesis. The fact that the oligonucleotide does not bind to the reverse clone also strongly suggests that this binding is specific to the *pyk 1* fragment and not to vector sequences.

Three theories were proposed to suggest why the oligonucleotide might bind to the DNA yet not prime de novo DNA synthesis;

1. The oligonucleotide is not binding to the DNA under the conditions for DNA sequencing, this may be because the oligonucleotide is too short and/ or not sufficiently GC rich to form a suitable primer for de novo DNA synthesis.

2. The 3' end of the oligonucleotide may be blocked in some way as to prevent polymerisation.

3. There may be a region of extensive secondary structure within the template which blocks polymerisation from this end of the gene.

Reports from other workers within and outwith our department confirm that oligonucleotides of this length and this complexity should be able to direct mutations. The commercially available M13 reverse sequencing primer is shorter, being a 17mer, and less GC rich than at least one of the failed oligonucleotides (35%) yet can still be used as an efficient sequencing primer suggesting that the problem is more complex than purely instability.

A report from the OSWELL DNA synthesis service, which provided all of the oligonucleotides used in this project, suggests that this first theory is unlikely.



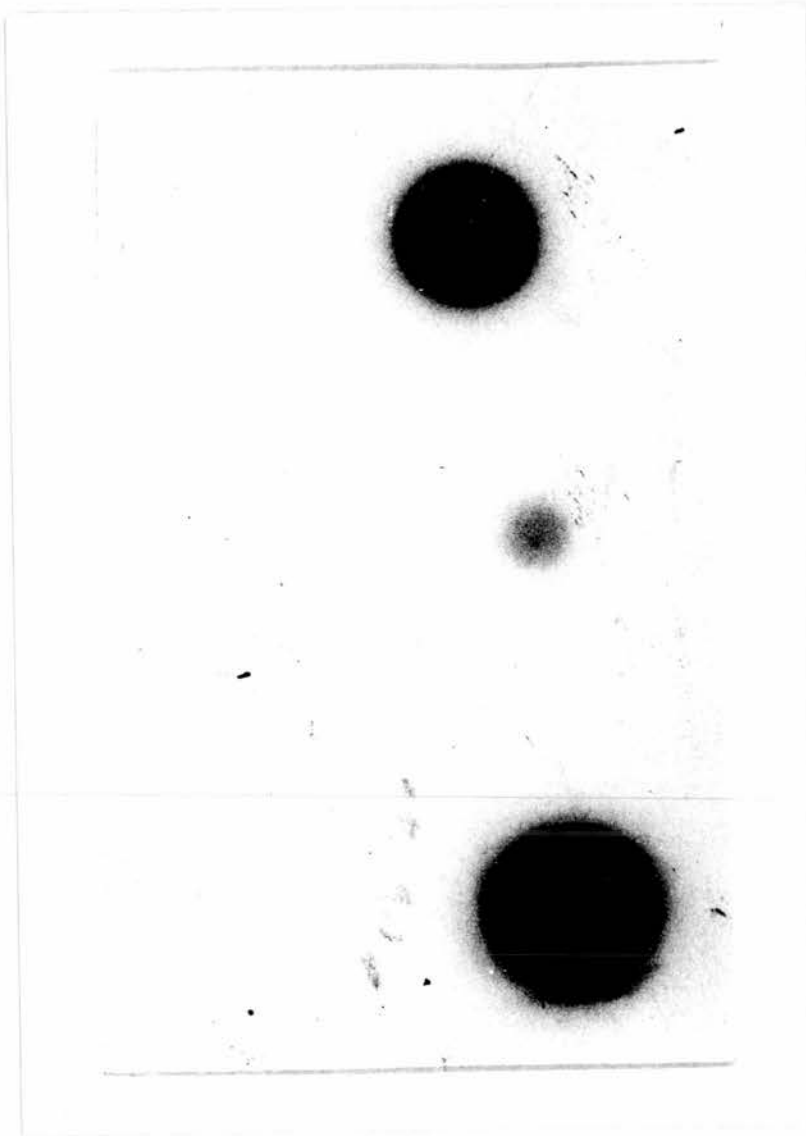


Figure 18; Dotblot of m13pyk single stranded DNA probed with the 19mer oligonucleotide which could not be used as a sequencing primer or to produce mutants. Dots 1 and 3 are m13ab1 which produces single stranded DNA in the opposite sense to the oligonucleotide, dot 2 is m13ab2 which is the same sense as the oligonucleotide, and is present as the non-specific binding control. 100ng of each clone were applied to the filter.

Oligonucleotides are synthesised in the 3' to 5' direction, the 3' end of the oligonucleotide being attached to a solid support. Once synthesis is complete the oligonucleotide is liberated by a simple hydrolysis reaction to yield the free 3' end. The chances of this simple process going wrong are said to be minimal. An HPLC elution profile of a small sample of the oligonucleotide used as a probe in the dot-blot shows that the synthesis has worked perfectly. The profile shows a single discrete peak migrating in the expected position of an unblocked 18mer. Were the 3' end of the oligonucleotide still blocked, this would not be the case.

This leaves the third theory, that there is an element of secondary structure downstream to the position of the bound oligonucleotide which prevents polymerisation. This may indeed be the case, but is very difficult to prove. Agarose gel mobility studies of the single stranded DNA template did not reveal anomalous mobility indicative of an extensive secondary structure. The UWGCG computer package includes a number of computer programmes designed to identify secondary structures within RNA. These programmes, such as mountains, squiggles and stemloops should be readily applicable to single stranded DNA molecules. Analysis of the pyruvate kinase template DNA using the programme STEMLOOP reveals a potential stemloop at the region of the template where the oligonucleotide binds, however there are also a large number of other potential stemloops within the DNA strand and the significance of this particular structure cannot be tested.

A new set of oligonucleotides were obtained. These were 29 base pairs in length. Their sequences are listed in the table below. These oligonucleotides were very effective as both sequencing primers and mutagenic oligonucleotides. The fact that these longer oligonucleotides were effective primers is evidence for the presence of an element of secondary structure



within the template DNA. Presumably the longer 3' end of the oligonucleotide possesses a stronger bonding energy, making the pairing of the template strand of the DNA more favourable than bonding of the template to itself.

Putative mutant plaques were selected for sequencing by single nucleotide tracking. The efficiency of mutagenesis with these oligonucleotides, using the Kunkel method of site directed mutagenesis, varied between 80-100%. This is comparable to the efficiencies reported using the Amersham site-directed mutagenesis kit, the most efficient mutagenesis method reported. A selection of the plaques shown to be mutant by single nucleotide tracking were sequenced using the sequenase DNA sequencing kit. An autoradiograph produced from such a sequencing reaction is illustrated in figure 19. The mutation K269R changes the wild type sequence "CAAGAT" to "CAGAAT", the region of the mutation is more than 200bp away from the sequencing primer and sequences located this far away from the primer becomes very difficult to read unambiguously. To circumvent this problem this mutant was also screened by differential hybridisation using the radioactive mutagenic oligonucleotide as a probe. The results of such a slotblot are shown in figure 20.

#### 6.1.4. SITE-DIRECTED MUTAGENESIS IN THE INTERSUBUNIT INTERFACE

With the initial problems surmounted and the sequence of the DNA around the intersubunit interface confirmed, an attempt was made to produce site-directed mutants within the intersubunit interface. Three mutations were made in total. These were all directed against serine 414, the residue that meets with itself across the 1,3 intersubunit interface as illustrated in figure 21. This serine was changed to an arginine, in the hope of setting up a charge repulsion which would destabilise the interface, and a histidine which might

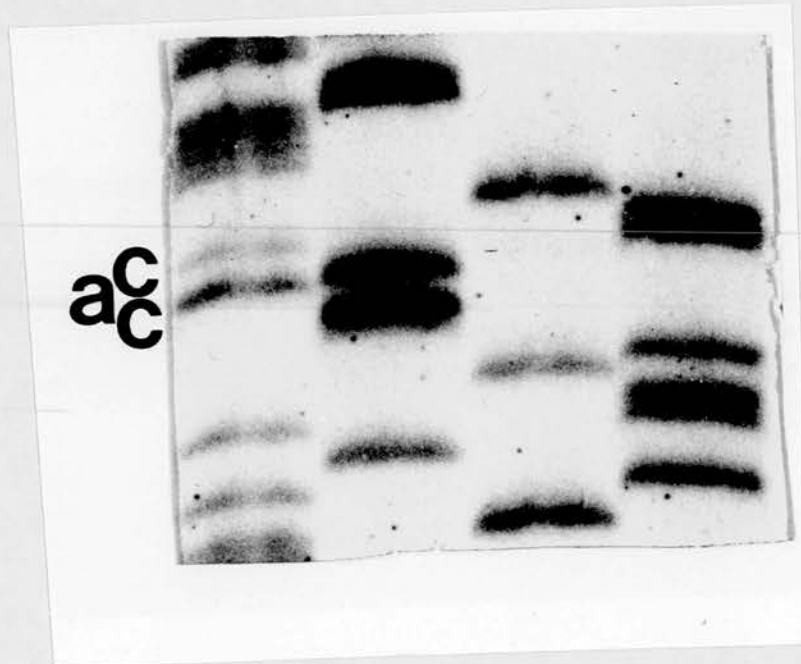
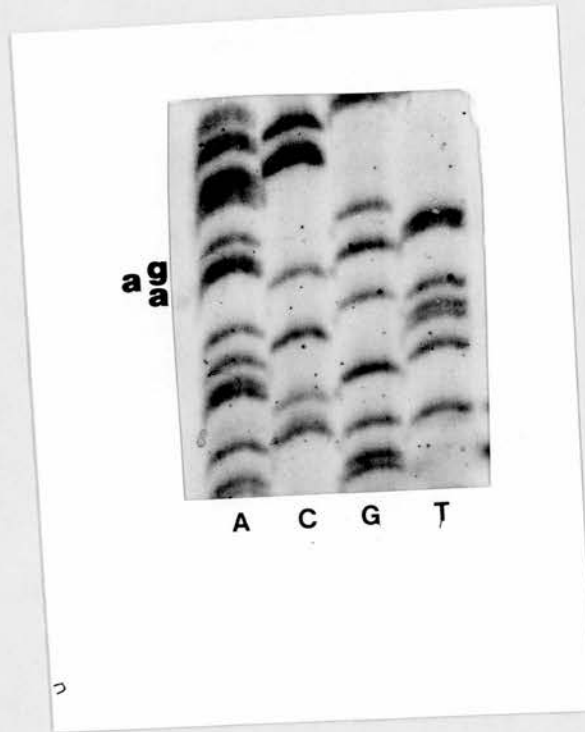


Figure 19; Sequencing autoradiographs of wild type and the K269H mutant.

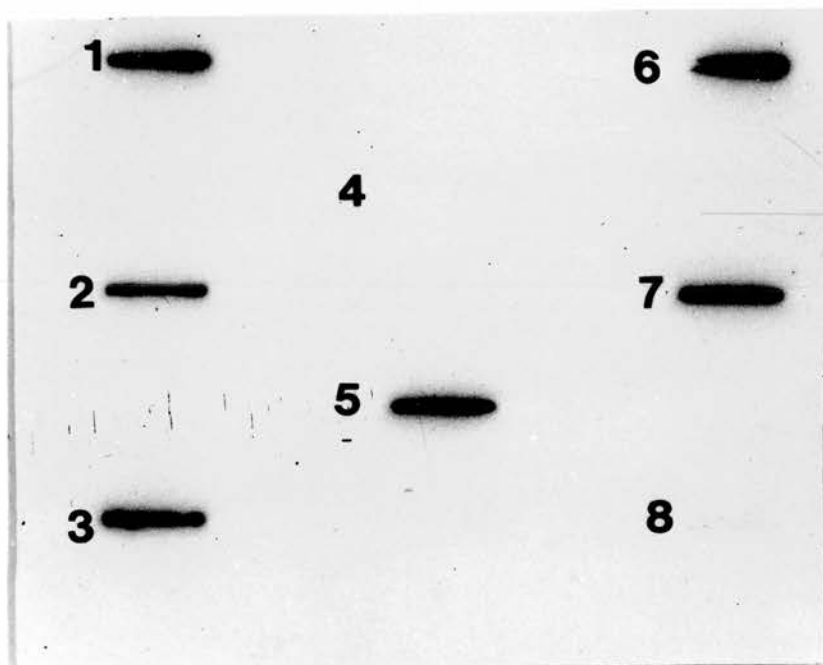
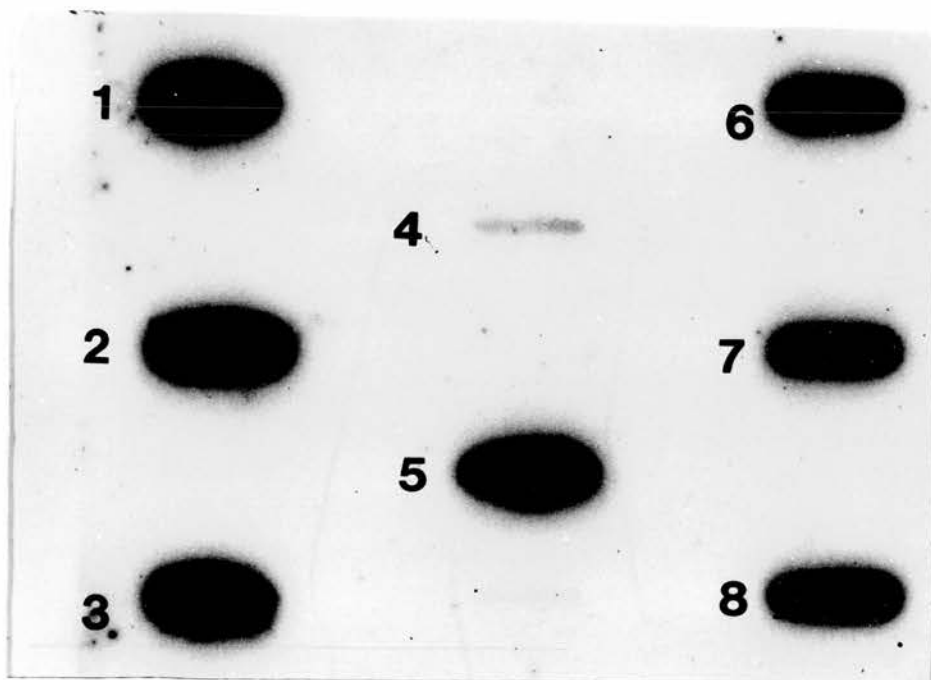


Figure 20; Slotblot of the K269R mutant. Slot 8 contains single stranded DNA prepared from wild type 13ab1 phage, slot 1,2,3,5,6 and 7 contain single stranded DNA from putative mutant plaques. Slot 4 is a negative control containing m13ab2 DNA which is in the same sense as the oligonucleotide. The upper figure illustrates an autoradiographic exposure of a filter washed in 1x SSC at room temperature, while the lower figure represents the same filter after a 0.1xSSC, 65° wash.

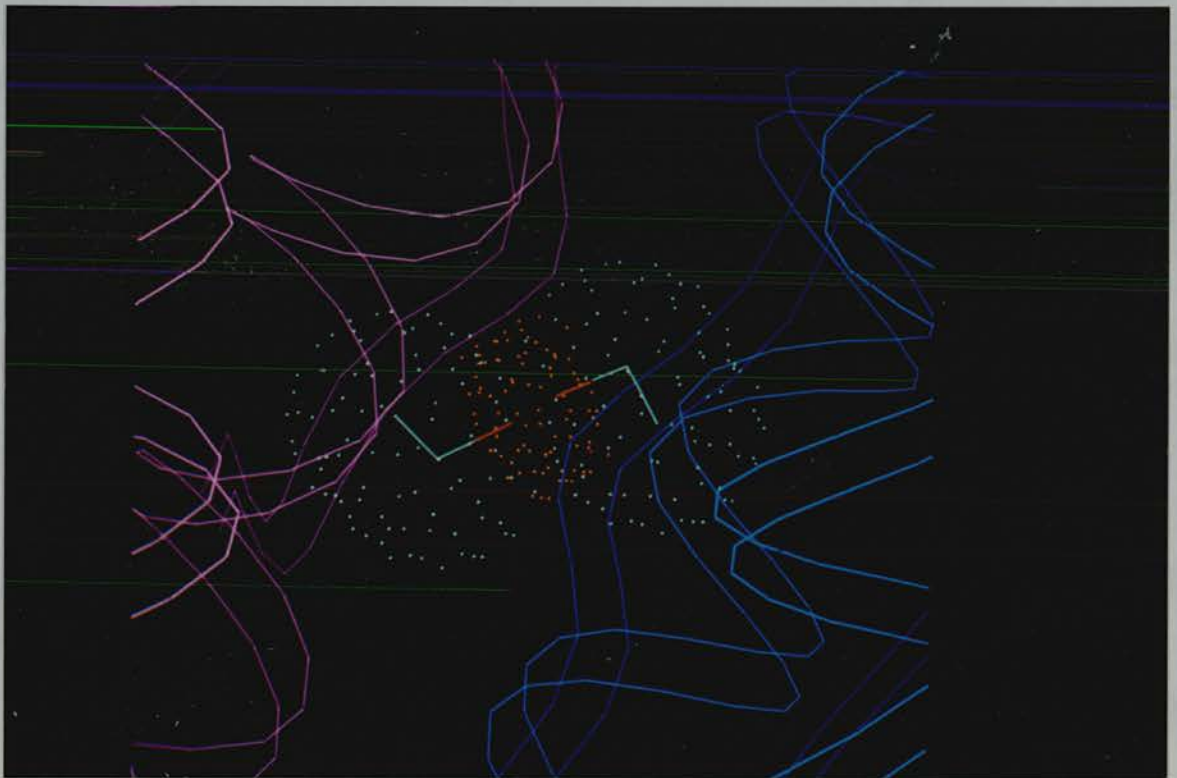
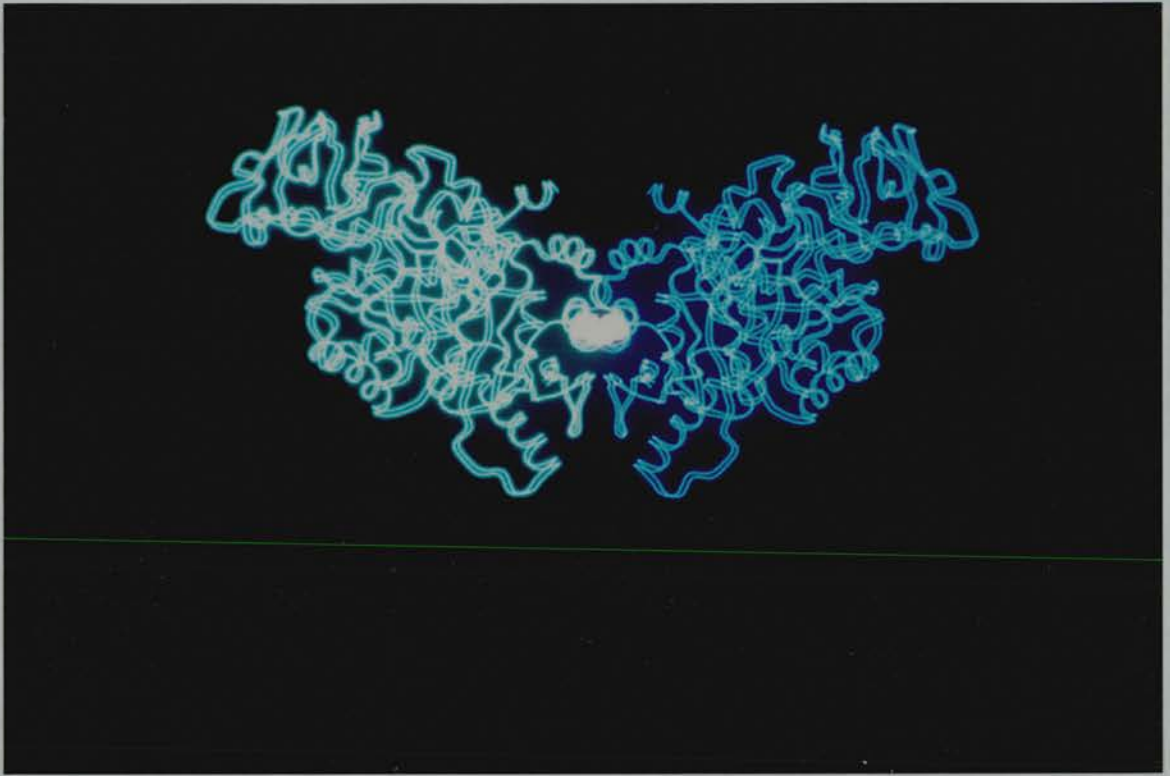


Figure 21: Plot of pyruvate kinase with the serine residues across the 1,3 intersubunit interface highlighted.

serve the same purpose were the side chain charged a physiological pH, and perhaps add an element of steric hinderance. This serine was also changed to a proline. While it is commonly accepted that proline, as an imino acid, cannot be accommodated within regular  $\alpha$  helices, there are examples where they are found, for example in the yeast phosphoglycerate mutase enzyme. The proline in this enzyme has the effect of making the helix kink slightly. It was hoped that the introduction of a proline residue into the C $\alpha$ 2 helix of pyruvate kinase may cause the helix to kink in such a way that it was no longer possible for the helices to form an effective interaction at the intersubunit interface. The autoradiographs of the sequencing of these mutant of pyruvate kinase are illustrated in figures 22-24.

#### 6.1.5. OLIGONUCLEOTIDES USED FOR MUTAGENESIS

MUTATION	OLIGONUCLEOTIDE	MISMATCH	LENGTH	%GC	T <sub>m</sub>
ACTIVE SITE					
WILDTYPE	CATTGTCAAGATTGAAAACCAACAAGGTG	-	-	-	-
1. K269H	CATTGTCCACATTGAAAAC	2	19	37	50
2. K269R	CATTGTGAGAATTGAAAAC	2	19	32	48
3. K269H	CATTGTCCACATTGAAAACCAACAAGGTG	2	28	43	58
4. K269R	CATTGTGAGAATTGAAAACCAACAAGGTG	2	28	39	57
INTERSUBUNIT INTERFACE					
WILDTYPE	CCGTCGCTGCCTCCGCTGTCGCTGCTG	-	-	-	-
5. S414R	CCGTCGCTGCCAGAGCTGTCGCTGCTG	3	27	70	69
6. S414H	CCGTCGCTGCCCACGCTGTCGCTGCTG	2	27	74	71
7. S414P	CCGTCGCTGCCCCAGCTGTCGCTGCTG	2	27	74	71
8. V411M	CGAAACCATGGCTGCC	-	16	63	-
9. A418T	GCCTCCGCTTGGGTTTTC	-	18	61	-

T<sub>m</sub> was determined by the formula  $69.5 + 0.41(\%GC) - 650/L$ , Oligonucleotides 1 and 2 did not direct mutations, while 8 and 9 were designed before errors in the published sequence had been corrected. Where specific mutations are referred to in the text, it is implied that these mutations were directed by oligonucleotides 3-7.



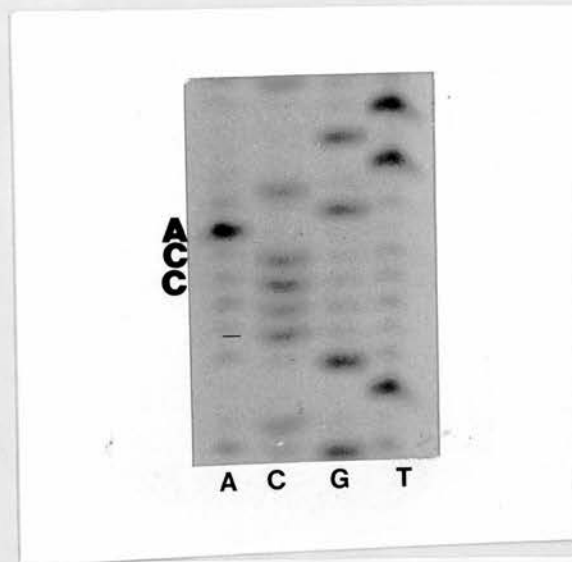


Figure 22; Autoradiograph of the mutant S414P. The wild type sequence is illustrated in figure 13. While the sequence of this mutant is quite clear, a few faint spurious bands are apparent, possibly as a result of the mutation which introduced two more 'C' residues into a region of the DNA which is already difficult to sequence due to its high 'GC' content.

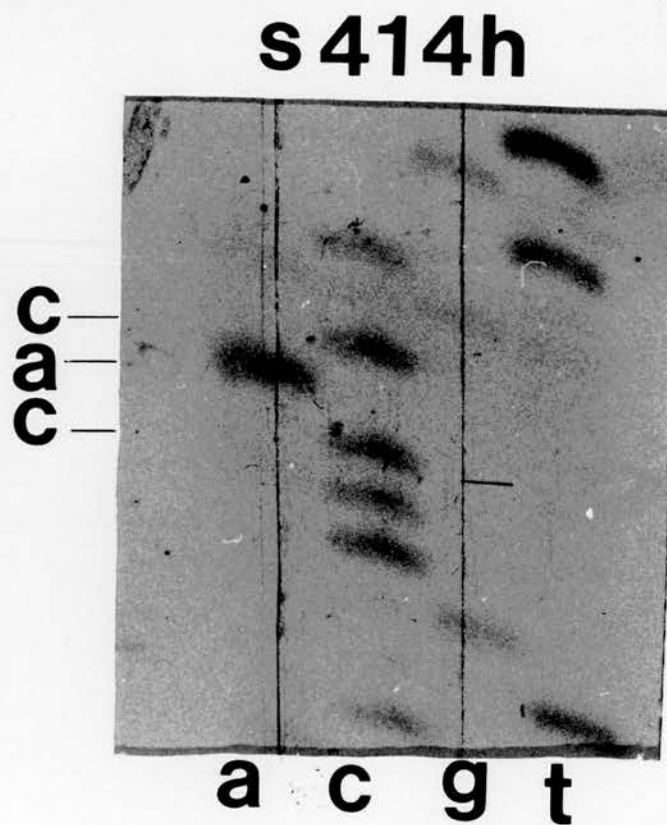


Figure 23; The mutation S414H

#### 6.1.6. Conclusions.

Initial attempts at site directed mutagenesis met with failure due to inaccurate publication of the original sequence data (Burke *et al.* 1983). While these errors were corrected, efforts at mutagenesis were directed at the active site of the protein. Two 19mer oligonucleotides were obtained to direct the mutations K269H and K269R. These oligonucleotides would not direct mutations within the active site, nor would they act as primers for the generation of sequence data by the dideoxy method. Longer oligonucleotides were obtained, and these served to direct mutations with high efficiency. The policy of using longer oligonucleotides was adopted for mutagenesis within the intersubunit interface, once the correct sequence had been confirmed. 5 mutations have been synthesised and verified. K269R and K269H are mutations of the putative catalytic lysine residue, while S414R, S414H and S414P are all directed to a serine residue within the intersubunit interface. The mutagenesis was performed on a 1.2 Kb fragment of the *pyk 1* gene, which was convenient for cloning into the single stranded phage vector M13. A number of subcloning steps, summarised in figure 25, were required before mutant protein could be produced.

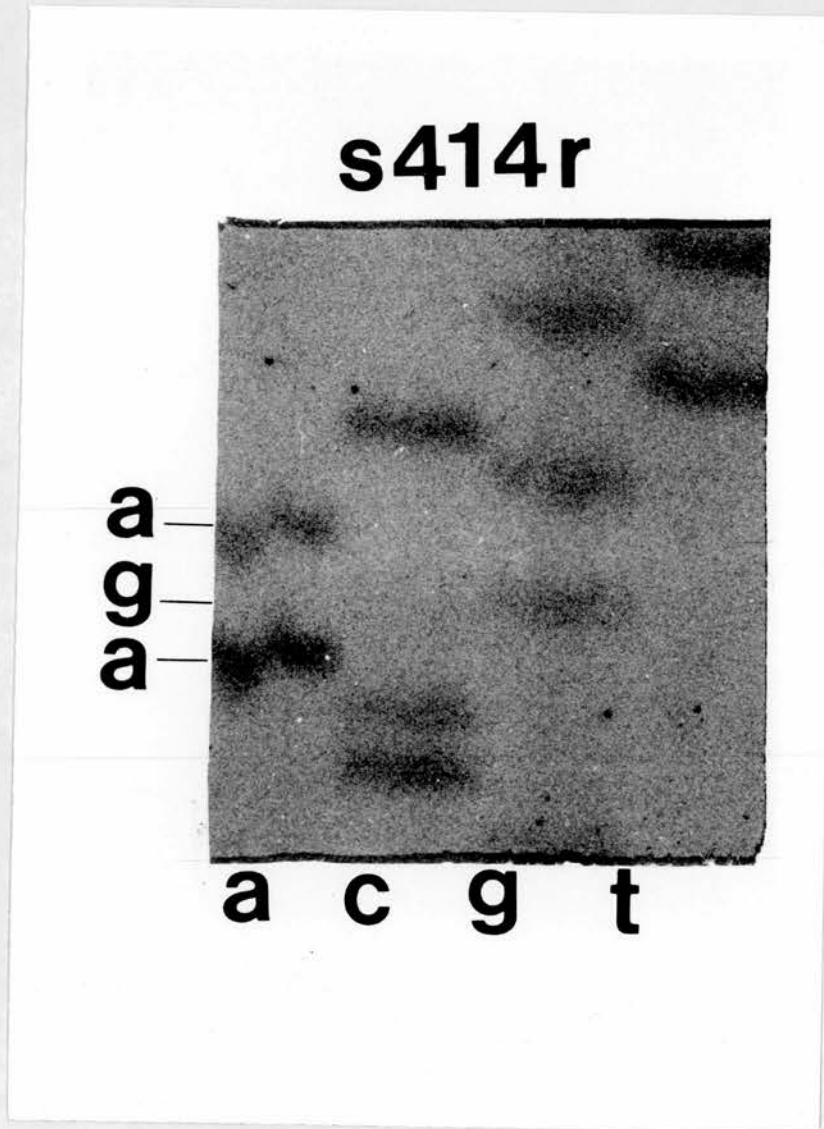


Figure 24 : The mutation S414R



## Chapter 7

### 7.1. SUBCLONING OF THE MUTATED DNA

#### 7.1.1. Production of mutant shuttle vectors

Only a small portion of the pyruvate kinase gene could be subcloned and mutated in the M13 vector as discussed previously. Therefore the mutated section of the gene had to be subcloned to produce the mutated gene in the expression vector pMA91pyk. A diagrammatic summary of this subcloning process is shown in figure 25.

RF DNA was prepared from the mutant M13 clones as described in the methods section of this thesis. The pyruvate kinase coding section of these clones was excised from the RF DNA using the EcoRI and HindIII site in the polylinker. This DNA was cloned into the pUC derived plasmid pK19, a small high copy plasmid bearing the kanamycin resistance gene. This plasmid was used as a source of the mutated BglII/BstEII fragment of the pyruvate kinase gene. The high copy number of the plasmid ensures a high yield of the fragment from CsCl gradients. This plasmid is easier to handle than M13RF DNA which gives low yield in CsCl preparations and is highly susceptible to recombination over prolonged growth periods.

The entire 7kb HindIII fragment of yeast DNA comprising the *pyk1* gene and surrounding sequences was cloned into the plasmid pUC18. This plasmid, pPYK20, is illustrated in the map in section 2 of this thesis. pPYK20 was cut to completion with the enzymes BglII and BstEII and the fragments separated by slow electrophoresis on a 1% agarose gel. This digestion yielded a fragment of 0.7kb which was the wild type BglII/BstEII fragment and a larger fragment of 8.8kb, which comprised the rest of the *pyk1* gene, surrounding sequences and

vector DNA. Great care was taken to separate these fragments as carefully as possible. DNA bands were excised from the agarose gel and purified as described in the methods section of this thesis. Controls were performed to ensure that the 8.8Kb fragment could not recircularise by ligating an aliquot of the DNA and transforming into competent bacterial cells. Only batches of the 8.8Kb fragment which, when transformed into competent cells, produced no bacterial colonies were used for subsequent ligation experiments.

Mutant BglII/BstEII fragments were produced by digestion of the appropriate pK19pyk clone with these enzymes and subsequent electrophoresis and purification as described previously.

These two DNA fragments were ligated together to form the relevant mutant pPYK20 clones.

In the early stages of the project the plan was to then subclone the 7kb HindIII fragments from the mutant pPYK20 clones into the yeast/E. coli shuttle vector pJDB207. This would permit the overexpression of the pyruvate kinase gene from its own promoter. However, in light of the evidence discussed in chapter 5, that this overexpression is transient, a new strategy was adopted. This involved the use of the expression vector pMA91pyk, which permits the overexpression of the *pyk1* gene from the yeast PGK promoter. A map of pMA91pyk is illustrated in fig 12 of this thesis. pMA91pyk was digested with the enzymes BglII and SstI to remove the wildtype pyruvate kinase sequences. The mutant pPYK20 plasmids were digested with these same enzymes to yield the mutated *pyk1* fragments. These were then ligated together to form the mutant pMA91pyk clones. Due to lack of time only the plasmids pMA91pykS414P and pMA91pykK269H were produced, however mutant pK19 DNA has been prepared for all 5 mutants and merely requires subcloning.

H  
E  
Bg  
Bs

HinDIII  
EcoRI  
BglII  
BstEII

Shaded areas represent the *pyk1*  
structural gene

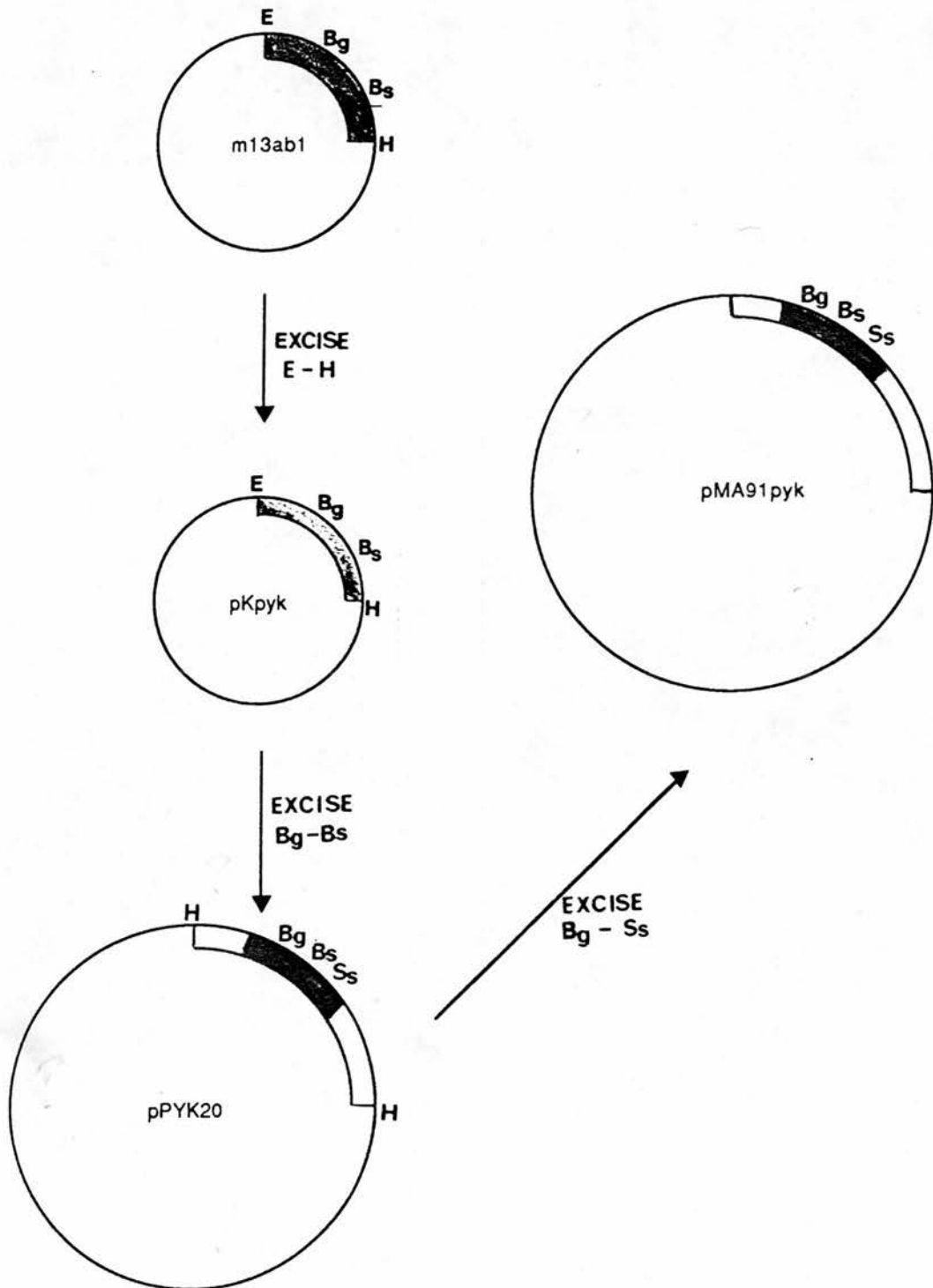


Figure 25, The subcloning procedure required to produce a mutated gene in an expression vector.

## 7.1.2. VERIFICATION OF MUTANT SHUTTLE VECTORS

The subcloning process described above required that at two stages wild type DNA be purified away from vector sequences. While careful controls were carried out at every stage possible to ensure that there was no cross contamination of mutant sequences with residual wild type, I felt it was necessary to further ascertain that the mutant shuttle vectors pMA91pykS414P and pMA91pykK269H were indeed mutant. There are three ways in which this could be done;

1. Double stranded plasmid sequencing
2. Differential hybridisation with the mutagenic oligonucleotide
3. Transformation of yeast, production of protein subsequent kinetic characterization and comparison to wild type.

In this instance double stranded DNA sequencing would only be possible for one of the mutants, pMA91pykS414P, as an oligonucleotide was available which would prime 5' to the mutated region to yield suitable DNA sequence information. There was no suitable primer available to screen the mutant pMA91pykK269H in this way. Thus it was decided to adopt the second method described above namely, differential hybridisation with the mutant oligonucleotide. This experiment gave very unsatisfactory results. Blots were prepared with BglII/BstEII digests of all clones. Each blot contained samples of DNA from the putative mutant shuttle plasmid, the relevant mutant pKpyk clone, the mutant RF DNA, wild type pMA91pyk and wild type pPYK20. In each case the oligonucleotide bound only to the 0.7Kb DNA fragment as expected, however it was impossible to obtain a differential removal of the oligonucleotide probe no matter how carefully the washing procedure was

carried out. At low stringency the oligonucleotide bound to all sequences with similar avidity, however increasing the stringency of washing to well below that approximated by calculation of the  $T_m$  of each oligonucleotide, they bound tenaciously to all samples with a similar avidity until eventually dissociating from the positive and negative controls and test samples at very low stringencies, 0.01xSSC, 70°C. This experiment was repeated four times, once by another worker in the laboratory, in an attempt to rule out operator error, however the same result was obtained in every case. The mutant M13ab1 clones were both resequenced and found give the correct mutant sequences as expected. The  $T_m$  of oligonucleotides is commonly calculated by the Wallace rule,

$$\begin{aligned} &\text{Dissociation temperature of an oligonucleotide in 1M Na}^+ \\ T_m &= 4 \times (\text{No. of GC base pairs}) + 2 \times (\text{No. of AT base pairs}) \end{aligned}$$

This gives an approximation of the dissociation temperature of oligonucleotides of between 12 and 23 base pairs and is not recommended for use with oligonucleotides outwith this size range. The oligonucleotides used in these experiments are 27mers and 29mers and thus are not governed by the Wallace rule. Another approximation of  $T_m$  for mismatch oligonucleotides is the formula

$$T_m = 69.3 + 0.41(G+C\%) - 650/L$$

where L is the length of the oligonucleotide in base pairs, bearing in mind that the  $T_m$  of a duplex decreases by 1°C with every increase of 1% in the number of mismatched base pairs.

Using these considerations, the  $T_m$  for the oligonucleotide S414P should be around 68°C in 1X SSC, and K269H around 56°C in the same salt conditions. The oligonucleotides were observed remain bound to the template at these temperatures in 0.1XSSC and in 0.01XSSC.

On reflection these oligonucleotides appear to be too long to be useful as

probes in this kind of differential hybridisation experiment. However, even though this method of screening was unsuccessful, an attempt was made to transform the putative mutant clones into yeast to examine any changes in the pyruvate kinase enzyme, the third characterisation method described above.

## 7.2. TRANSFORMATION OF MUTANTS INTO YEAST

### 7.2.1. Production of transformants

While the results of the differential hybridisation were unsatisfactory, all the evidence from the subcloning controls suggests that the final product of the subcloning is very likely to be mutant. Thus it was decided to proceed with these clones and transform them into the disrupted yeast strain. Yeast transformations were performed by the method of Ito as described in the methods section of this thesis. All transformations were set up in the presence of a negative control comprising yeast cells with no DNA added. Plates were incubated at 30°C under selective conditions to allow only the growth of transformed yeast colonies. In the case of the transformation of the putative mutant yeasts, it took 20 days for colonies to appear on the growth plates. No colonies grew on the negative control plates with no DNA added. S414P produced 120 colonies and K269H, 200 colonies for approximately 5µg of DNA. These transformation efficiencies are rather low compared to those reported in the literature, however yeast transformation efficiency is known to vary greatly with different batches of polyethyleneglycol and transformation of plasmids bearing the *leu2d* allele is always very inefficient by this particular method.

Unfortunately it has proved impossible to subculture any of the mutant transformants obtained. There was no time to look into this further.

## Chapter 8

### 8.1.PROTEIN PURIFICATION

#### 8.1.1. Purification of pyruvate kinase from *Saccharomyces cerevisiae*

Pyruvate kinase has been purified from a great many sources (Guderley *et al.* 1989) including the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* (Yun *et al.* 1976, Roschlau *et al.* 1972, Haeckel *et al.* 1968). Most methods utilise an affinity purification procedure using Cibacron-blue, an affinity matrix which binds ADP binding proteins. The dye Cibacron-blue is relatively inexpensive to buy, but affinity purification matrices such as Cibacron-blue bound to Sepharose are very expensive. For this reason we synthesised our own Cibacron-blue Sepharose in the laboratory.

#### 8.1.2. Synthesis of Cibacron-blue sepharose-6B

A number of methods have been published describing the attachment of cibacron-blue dye to chromatography matrices such as Sephadex, cellulose and Sepharose. Sepharose was used in this case because of the ease of coupling of the dye and the matrix, in that this did not require preincubation with noxious chemical agents such as cyanogen-bromide. Cibacron-blue can be rapidly and efficiently coupled to the matrix by incubation at high pH in high salt as described in the methods section of this thesis. Unfortunately this home made Cibacron-blue Sepharose was extremely heterogeneous regarding its affinity for pyruvate kinase so a number of batches were synthesised before one was deemed suitable for use. This particular batch retained 80% of the total pyruvate kinase on the column initially, however this value dropped to around 30% of the total sample over a period of two months. It is likely that the column was becoming fouled by microorganisms. Pyruvate kinase is



reported to be cold sensitive thus all purifications were carried out at room temperature, this greatly facilitated the growth of microorganisms. Although all buffers were treated with 0.05% sodium azide, this compound is light sensitive and thus rapidly degraded. The components of the elution buffer used for the cibacronblue column namely fructose-1,6-bisphosphate and phosphoenolpyruvate are excellent substrates for microbial growth.

Elution of the pyruvate kinase from the Cibacron-blue Sepharose using the specific elutants, fructose-1,6-bisphosphate and phosphoenolpyruvate as described gave a homogeneously pure preparation of the enzyme as determined by coomassie blue staining (Fig 17). The enzyme is produced at a much higher specific activity than previously reported, a reflection of the advantages of protein production from an overproducing strain.

Another factor is undoubtedly the fact that the enzyme is eluted from the column in the presence of fructose-1,6-bisphosphate, an allosteric activator of the enzyme. Although buffer containing the enzyme was exchanged by amicon filtration, residual traces of fructose-1,6-bisphosphate would be expected to alter the kinetics of the enzyme drastically.

An alternative method of purification of the enzyme was sought, which did not rely on affinity elution from Cibacron-blue Sepharose. The anion exchange medium, Q Sepharose was utilised on the advice of Dr. T. Murcott of the department of biochemistry, University of Bristol. The purification protocol is similar to that described in section 2.10 of this thesis, except that the cibacron-blue chromatography step is replaced by a Q sepharose step in which the protein is equilibrated with 50mM Tris-Cl pH 8.5, 50mM KCl, 5mM EDTA, 0.05mM PMSF, and 0.1mM Benzamidine. Pyruvate kinase elutes with the breakthrough volume, and over 85% of the activity applied to the column is recovered. The protein is recovered greater than 90% pure as determined by



SDS-PAGE. The kinetic parameters were determined to be;  $K_m$ PEP 4mM,  $K_m$ ADP 1mM, and SA 382 units per mg protein.

There are several advantages to using the pyruvate kinase produced by this method, even over that which is purer from the affinity elution procedure. Cibacronblue binds many proteins, mostly those that bind ATP or fructose compounds. Trace amounts of these proteins in the pyruvate kinase preparation, even in concentrations so small as to not be discernable by coomassie staining, may significantly skew kinetic analyses, if the contaminating protein had a higher affinity for ADP for example. Q Sepharose binds proteins on the basis of their ionic charge, although a few minor proteins are observed to copurify with pyruvate kinase using the protocol described, these are unlikely to have common substrate binding properties and thus are unlikely to interfere in kinetic analyses of enzyme activity.

The protein produced by the Q Sepharose method appears to be stable. Pyruvate kinase from *Saccharomyces cerevisiae* is notoriously unstable, especially in the presence of fructose-1,6-bisphosphate and in the cold (Hunsley and Sealter, 1969, Yun *et al.*, 1976). Protein produced by the cibacron-blue method, while appearing to be pure, lost at least 25% of its enzymic activity within 2 weeks of purification. SDS-page analysis of a sample of the protein which had incurred a reduction of activity upon storage showed that the protein ran similarly to a freshly prepared preparation suggesting that the loss of activity was not as a result of proteolytic degradation of the enzyme (fig 17).

## chapter 9

### CONCLUSION

The investigation of the allosteric nature of pyruvate kinase is an extensive project and could not be expected to be completed within the limited timespan of a single PhD project. The work presented here describes the setting up of an effective system for these studies. This preliminary work proved to be much more time consuming than originally planned, due to a number of problems encountered during the course of this project. The first hurdle encountered was the error in the published sequence of the protein. This affected the progress dramatically as the error occurred in the region of the gene which encodes the 1,3 intersubunit interface. Problems were also encountered when trying to mutate outwith this region, however these were overcome by the use of longer mutagenic oligonucleotides. Overexpression of pyruvate kinase also proved to be less straightforward as originally anticipated due to the dosage compensation and other regulatory effects observed when the protein is expressed by its own promoter. This problem has now been alleviated with the use of the fusion product of the *pgk* promoter and the *pyk1* structural gene. This work is to be continued by Mr. Richard Collins, who should find it relatively easy, following the procedures outlined here, to express the mutant pyruvate kinases described herein, and then examine the effects of the mutations on the kinetics of the enzyme.

Expression and purification of the active site mutants described in this thesis should identify the essential residues within the active site of the protein such that the catalytic mechanism may be affirmed unambiguously.

There is still a lot of scope for mutagenesis of the C $\alpha$ 1 and C $\alpha$ 2 helices to assign their role in the allosteric transition. In recent years saturation

mutagenesis methods have become increasingly popular and this technique could lend itself particularly well to these studies. Saturation mutagenesis of the C $\alpha$ 1 and the C $\alpha$ 2 helices followed by transformation into the *pyk<sup>-</sup>* strain of yeast would reveal those mutations which yielded a functional pyruvate kinase protein. This could then be purified on a small scale to attest its sensitivity to allosteric effectors.

It may be the case that the C $\alpha$ 1 and C $\alpha$ 2 helices of the protein are not solely responsible for the allosteric nature of the yeast enzyme. It is therefore necessary to identify other important residues or regions of the protein in this respect, and perhaps subject them to mutagenesis.

The role of the N terminus in the regulation of other forms of the enzyme should also be investigated. The yeast enzyme has a truncated N terminus when compared to all other known forms. The N terminus is known to be the site of phosphorylation of the liver isoenzyme, and this phosphorylation event is known to have effects on the catalytic activity of the enzyme (section 1.1.3.). It would be interesting to alter the N terminus of the yeast enzyme by fusing on additional residues to make it more similar to the N-termini of some of the mammalian isoenzymes, and to analyse any effect on the kinetics of the enzyme. It may also be possible to swap N terminal domains of other pyruvate kinases and attest the effects of this on the kinetics of the enzyme. Clearly there is a great deal of work to be carried out, using pyruvate kinase as a model for allosteric control now that the groundwork required for the efficient production of mutant proteins has been completed

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## APPENDIX

# The yeast pyruvate kinase gene does not contain a string of non-preferred codons: revised nucleotide sequence

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The sequence of the gene encoding pyruvate kinase from *Saccharomyces cerevisiae* was re-determined because of failures with oligonucleotide-directed mutagenesis experiments involving a region thought to contain a string of five contiguous non-preferred codons. This region was found to be difficult to sequence and was shown to have three extra bases when compared with the published sequence [(1983) J. Biol. Chem. 258, 2193-2201]. The revised sequence demonstrates that the yeast pyruvate kinase gene does not have a cluster of non-preferred codons, and that it therefore is not an example of the class of genes which possibly exhibit translational control by the presence of non-preferred codons.

Pyruvate kinase gene; Nucleotide sequence; Translational control; Non-preferred codon; (*Saccharomyces cerevisiae*)

## 1. INTRODUCTION

Pyruvate kinase has been extensively studied because of its abundance in most organisms and because of its importance in glycolysis for controlling the flux from fructose 1,6-bisphosphate through to pyruvate. The enzyme catalyses the essentially irreversible conversion of phosphoenolpyruvate to pyruvate by the addition of a proton and the loss of a phospho group, which is transferred to ADP. The high resolution crystal structure of mammalian muscle pyruvate kinase has been determined and correlated with the protein sequence [1]. Amino acid sequences deduced from DNA sequences are also available for all four

mammalian isoenzymes [2-61], and for pyruvate kinase from chicken [7] and from *Saccharomyces cerevisiae* [8].

A notable feature of the yeast pyruvate kinase DNA sequence previously reported [8] is that it has an unusual arrangement of five consecutive non-preferred codons (as defined by Bennetzen and Hall [9]) located approximately four-fifths of the way through the coding region. This provided indirect evidence for the possibility that the gene may possess a translational pause in this region, thus enabling the multidomain structure of the enzyme to fold correctly during translation [10]. Experiments were devised by the Glasgow group to test this hypothesis by site-directed mutagenesis to replace the non-preferred codons with the synonymous preferred codons. Three oligonucleotides were synthesised for this purpose, but they failed to give the appropriate mutants, and their  $T_m$  values seemed to be lower than theoretically expected [11]. By coincidence, site-directed mutagenesis experiments involving the same region were being done by the Edinburgh group to study intersubunit contacts and allosteric control of pyruvate kinase. It was decided to re-examine the DNA sequence in

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The nucleotide sequence presented here will appear in the EMBL/GenBank database under the accession no. X14400

this region, and eventually to re-determine the entire sequence.

## 2. EXPERIMENTAL

The plasmid pAYE4(34) (fig.1), which corresponds to the *S. cerevisiae* *PYK1* gene cloned into the multicopy vector pJDB207 [12], was generously provided by R. Cafferkey, A. Goddey and B. Carter formerly of G.D. Searle. Suitable restriction fragments were subcloned into phage M13 for sequence determination by the dideoxy method. The oligonucleotides used by the Glasgow group were synthesised by V. Math, A.J.P. Brown and J.R. Coggins (Departments of Biochemistry and Genetics, University of Glasgow); those used by the Edinburgh group were from Oswel DNA Service, University of Edinburgh.

Site-directed mutagenesis was done by the Glasgow group in an attempt to replace the contiguous string of five non-preferred codons in *PYK1* in the proposed 'pause region' [10] with the synonymous preferred codons. The procedures of Winter et al. [13] were used. Following mutagenesis, phages were screened by plaque hybridisation with the <sup>32</sup>P-end labelled mutagenic oligonucleotide as a probe. Filters were serially washed at increasing temperatures, and autoradiographed between each wash [14]. The expected *T<sub>m</sub>* value for the wild-type *PYK1* sequence was 37°C, whereas that for the mutant was 74°C. In fact, the plaques displayed a range of *T<sub>m</sub>* values intermediate between these two extremes. Several putative mutant phages were sequenced with the use of a 14-mer primer of the sequence 5'-TGTACTCCAAAGCC-3' which hybridises 16 nucleotides upstream from the 'pause region'. To our surprise the sequences neither contained the published wild-type nor the expected modified sequences. Instead, all contained three extra C-residues interspersed with the wild-type 'pause' sequence (fig.2). Sequencing of the original plasmid pAYE4(34) in this region established that the sequence differences did not arise during the multiple subcloning steps during the mutagenesis experiments. The Edinburgh group independently confirmed the presence of the extra C-residues with a different sequencing primer.

## 3. RESULTS AND DISCUSSION

### 3.1. Nucleotide sequence of *PYK1* gene

The strategy used to sequence the *PYK1* gene is summarised in fig.1. A contiguous stretch of 1791 bases was sequenced which includes an open reading frame of 1500 bases encoding the enzyme pyruvate kinase (fig.2). The sequence is identical to the previously published sequence [8] except for three extra C-residues located between codons 379 and 386. These sequence changes have the consequence that the five non-preferred codons are converted to six preferred codons.

Both the Glasgow and Edinburgh groups found that the results of DNA sequencing reactions in this region were unusually difficult to interpret (fig.3). This was true with the use of different primers and

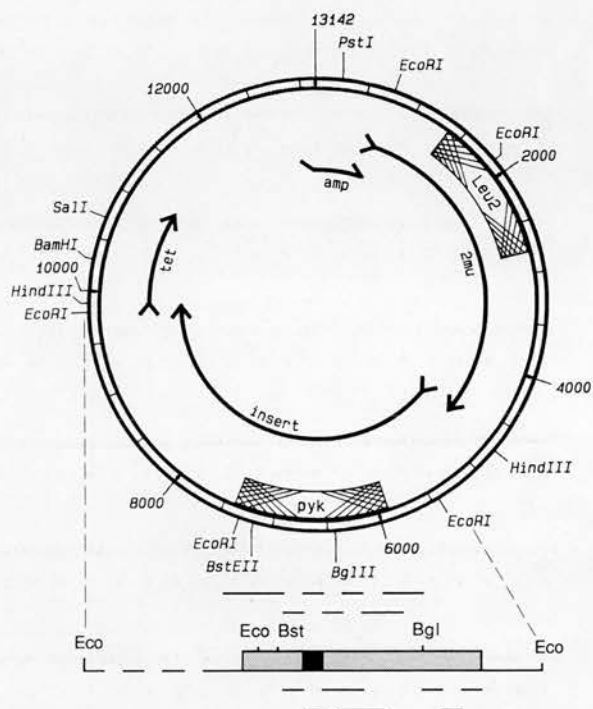


Fig.1. The yeast/*E. coli* shuttle plasmid pAYE4(34) with a 7 kbp insert encoding the yeast *PYK1* gene. The two *EcoRI* restriction fragments covering the entire *PYK1* coding sequence were subcloned individually into pAT153 and subsequently into M13mp19 in two orientations to allow sequencing of both strands. In addition, the *BglII/EcoRI* fragment containing the sequence differences was cloned into M13mp18 and M13mp19 to allow for detailed analysis of the region. The contig diagram illustrates the sequencing strategy. Universal sequencing primers were used, as well as oligonucleotides synthesised for use in the mutagenesis studies. The shaded region shows where the three extra 'C'-residues are located. This region of the DNA has been sequenced extensively on both strands using either the Klenow fragment of DNA polymerase or Sequenase.

with different DNA polymerases. It seems likely that these residues were missed with the sequencing conditions used by Burke et al. [8]. An additional and different line of evidence in support of this suggestion comes from the fact that the revised sequence is more similar to pyruvate kinase sequences from other organisms (fig.4).

### 3.2. Translational pauses specified by non-preferred codons

It is clear from these results that pyruvate kinase no longer provides strong indirect evidence to support a possible link between the rate of mRNA



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AAATGCTAGATTAGAAAGATTGACCTCATTAAACGTTGTTGCTGGTTCTGACTTGAGAAAGAACCTTCATCATTGGTACCATCGGTCCAAAGACCACCAACCCAGAAACCTTGGTTGCTTT
1 10 20 30
M S R L E R L T S L N V V A G S D L R R T S I I G T I G P K T N N P E T L V A L -

GAGAAAGGCTGGTTTGAACATTGTCCTGATGATCTCTCTCAGGTTCTTACGAATACCAAGTCTGTCATTGACAACGCCAGAAAGTCCGAAAGATTGTACCCAGGTAGACCATTTGSC
40 50 60 70
R K A G L N I V R M N F S H G S Y E Y H K S V I D N A R K S E E L Y P G R P L A -

CATTGCTTTGACACCAAGGTCAGAAATCAGAACTGTTACCACCAACGATGTTGACTACCCAATCCCACCAACCAAGAAATGATCTTACCACCGATGACAAGTACGCTAAGGC
80 90 100 110
I A L D T K G P E I R T G T T T N D V D Y F I P P N H E M I F T T D D K Y A K A -

TTGTGACGACAAGATCATGTACGTTGACTACAGAACATCACCAAGGTCATCTCCGCTGGTGAAGATCATCTACGTTGATGATGGTGTGTTTGTCTTCCAAGTTTGGAAAGTCGTTGACGA
120 130 140 150
C D D K I M Y V D Y K N I T K V I S A G R I I Y V D D G V L S F Q V L E V V D D -

CAAGACTTTGAAGGTCAGGCTTTGAACGCCGGAAGATCTGTTCCACAGGCTGTAACCTTACCAGGTACCGATGTCGATTGCCAGCTTTGCTGAAAAGGACAAGGAAGATTGAG
160 170 180 190
K T L K V K A L N A G K I C S H K G V N L P G T D V D L P A L S E K D K E D L R -

ATTCGGTGTCAAGAACGGTGTCCACATGGTCTTCTGCTTCTTTCATCAGAACGCCAACGATGTTTACCATCAGAGAAGTCTTGGGTGAACAGGTGAAGGACGTCAAGATCATTGTCAA
200 210 220 230
F G V K N G V H M V F A S F I R T A N D V L T I R E V L G E O G K D V K I I V K -

GATTGAAACCAACAGGTTGTAACAACTTCGACGAAATCTTGAAGGTCAGTACGCTGTTATGTTGCCAGAGGTGACTTGGGTATTGAAATCCAGCCCCAGAGTCTTGGCTGTCCA
240 250 260 270
I E N O O G V N N F D E I L K V T D G V M V A R G D L G I E I P A P E V L A V Q -

AAAGAAATGATTGCTAAGTCTAAGTGGCTGGTAAAGCCAGTATCTGCTACCCAAATGTTGGAATCCATGACTTACACCAAGACCAACCAAGAGCTGAAGTTTCCGATGTCGGTAA
280 290 300 310
K K L I A K S N L A G K P V I C A T O M L E S M T Y N P R P T R A E V S D V G N -

CGCTATCTTGGATGGTCTGACTGTGTTATGTTGCTGGTGAACCCGCAAGGTAACCTACCAATCAACGCCGTTACCACATGCTGAAACCGCTGTCTTGTCTGAACAAAGCTATLGC
320 330 340 350
A I L D G A D C V M L S G E T A K G N Y F I N A V T T M A E T A V I A E Q A I A -

TTACTTGCCAAACTACGATGACATGAGAACTGTACTCCAAAGCCAACTCCACCACCGAAACCGTGCCTGCCCTGCTGCTGCTGTTTTCGAACAAAGGCCAAGGCTATCATTGT
360 370 380 390
Y L P N Y D D M R N C T F K F T S T T E T V A A S A V A A V F E Q K A K A I I V -
T S L P R -

CTTGTCCACTTCCGGTACCACCCCAAGATTGGTTTCCAAGTACAGACCAACTGTCCAATCATCTTGGTTACCAGATGCCAAGAGCTGCTAGATTCTCTCACTTGTACAGAGGTGCTTT
400 410 420 430
L S T S G T T F R L V S K Y R P N C P I I L V T R C P R A A R F S H L Y R G V F -

CCCATTCGTTTTCGAAAAGGAACCTGTCTCTGACTGGAGTATGATGTTGAAGCCGCTATCAACTTCGGTATTGAAAAGGCTAAGGAATTCCGGTATCTTGAAGAAGGCTGACACTTACGT
440 450 460 470
P F V F E K E P V S D W T D D V E A R I N F G I E K A K E F G I L K K G D T Y V -

TTCCATCCAGGTTTCAAGGCCGGTGTGGTCACTCCACACTTTTGAAGTCTCTACCGTTTAAAAAAGAAATCATGATTGAATGAAGATATTATTTTTTGAATTATTTTTTAAAT
480 490
S I O G F K A G A G H S N T L O V S T V

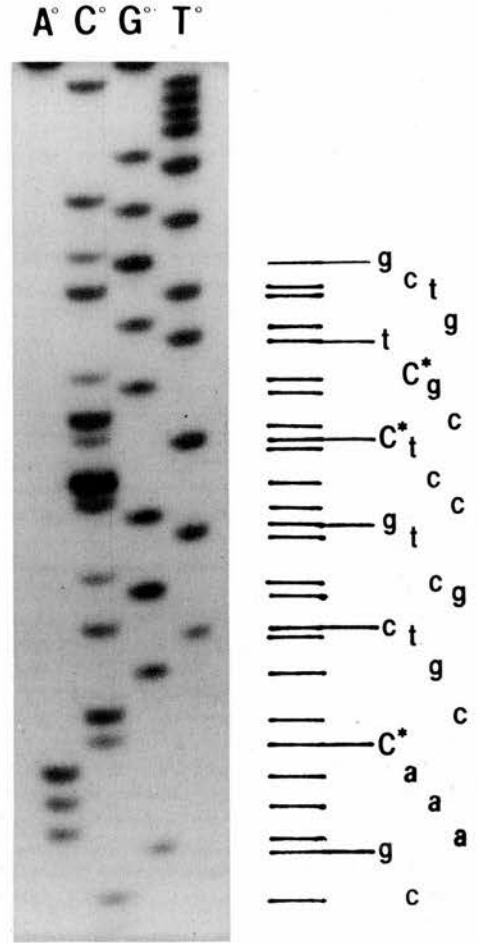
TTATATAAGACATGGTTTTCTTTTCAACTCAAATAAGATTTATAAGTTACTTAATAACATACATTTTATAAGGATTTCTATAAAAGATAACTTATGTTATTGTTAACTTTTTTG
CTCCAATTGTCGTCATACGATGAAGGTTTCGATTTTGGAAACGAGATTGACATAGAGTCAAAATTTGCTAAATTTGATCCCTCCCATCGCAAGATAATCTCCCTCAAG

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Fig.2. Nucleotide sequence and deduced amino acid sequence of *PYK1*. The three extra 'C'-residues found in this study are indicated by the asterisks, and are discussed in the text.



Fig.3. The results of dideoxy sequencing reactions with the Klenow fragment of DNA polymerase in the region of the sequence differences. The three 'C'-residues not reported in the original sequence data are indicated by the asterisks, and appear fainter than other C-residues in the same region. The use of dITP instead of dGTP had no effect on this observation. Sequenase was only effective for sequencing this region when 7-deaza-dGTP was substituted for dGTP in the reaction mixes.



translation and protein folding in vivo. However, this does not mean that the hypothesis is no longer tenable. The codon usage patterns in several other yeast genes reveal significant contiguous strings of non-preferred codons; these include *ARO1*, *GAL4* and *TRP3* [10] as well as *FAS1*, *HIS4* and *TRP5* (fig.5). In some genes the strings of non-preferred codons seem to be preferentially located close to interdomain regions (*ARO1*, *FAS1*), whereas in others no such correlations exist (*GAL4*, *HIS4*, *TRP5*). In the latter cases it is possible that the strings of non-preferred codons promote intradomain folding, but there is no evidence to support this. It remains attractive to suggest that putative translational pauses within the *ARO1* and *FAS1* genes temporally separate the synthesis, and hence the folding, of their multiple structural domains in vivo. As far as we know there is no direct evidence yet that either invalidates or supports the hypothesis. The Glasgow group is attempting to provide such evidence by mutating the string of ten non-preferred codons in *TRP3* [10].

# PYK

	370	380	390
Yeast [8]	... C T P K P T S T T E T S L P R - V A A V F E Q K A K A ...	* * * * *	
Yeast - this report	... C T P K P T S T T E T V A A S A V A A V F E Q K A K A ...		
Chicken M [7]	... H S V H H R E P A D A M A A G A V E A S F K C L A A A ...		
Cat M1 [1]	... G S S H S T D L M E A M A M G S V E A S Y K C L A A A ...		
Rat M1 [2]	... A S S Q S T D P L E A M A M G S V E A S Y K C L A A A ...		
Rat M2 [2]	... L A P I T S D P T E A A A V G A V E A S F K C C S G A ...		
Rat L & R [3-5]	... A A P L S R D P T E V T A I G A V E A S F K C C A A A ...		
Human L [6]	... A A P L S R D P T E V T A I G A V E A A F K C C A A A ...		

Fig.4. Pyruvate kinase amino acid sequences in the region of the yeast sequence differences (indicated by the asterisks). The yeast sequences are compared with those from chicken muscle [7], cat skeletal muscle isoenzyme M1 [1], rat skeletal muscle isoenzyme M1 [2], rat kidney isoenzyme M2 [2], rat liver and erythrocyte isoenzymes L and R [3-5], and human liver isoenzyme L [6]. Residues that are identical in four or more sequences are boxed.

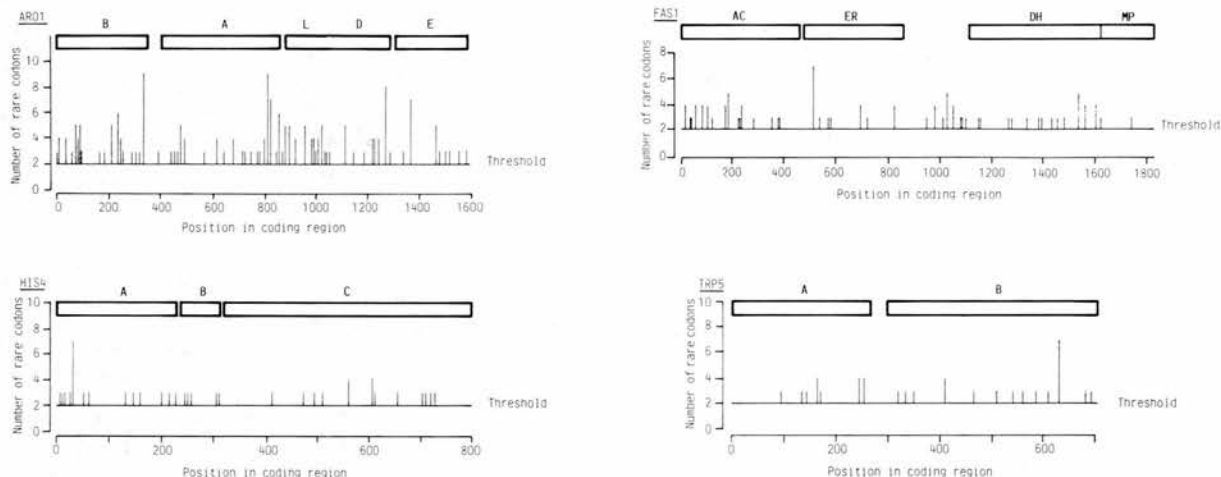


Fig.5. The distribution of non-preferred codons in the *ARO1*, *FAS1*, *HIS4* and *TRP5* genes from *S. cerevisiae*. The unique codons for methionine and tryptophan are considered to be neutral in this analysis; they do not stop a string of non-preferred codons, nor contribute to it. The number of consecutive non-preferred codons in a string is plotted against the location of the string within the coding region. The domain organisation of each coding region is presented above each graph. *ARO1* (diagram adapted from [10]; sequence published in [15]). *FAS1*: AC, acetyl transferase; ER, enoyl reductase; DH, dehydratase; MP, malonyl palmityl transferase [16]. *HIS4*: A, phosphoribosyl-AMP cyclohydrolase; B, phosphoribosyl-ATP pyrophosphohydrolase; C, histidinol dehydrogenase [17]. *TRP5*: A and B, alpha and beta domains of tryptophan synthase [18].

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## Site-directed mutagenesis as a tool for the study of the allosteric control of pyruvate kinase

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Pyruvate kinase (EC 2.7.1.40) catalyses the reaction



This is the second ATP-forming reaction of glycolysis and is considered to be a major control point for the lower end of the glycolytic pathway. In most species studied the enzyme is a homotetramer of  $M_r$  approx. 240 000; however, recent reports [1, 2] suggest that the enzymes from *Zymomonas mobilis* and *Schizosaccharomyces pombe* may be dimeric.

In mammalian systems four isoenzymes of pyruvate kinase are expressed in a tissue-specific manner. The L, R and M2 isoenzymes from liver, red blood cell and kidney, respectively, are all allosterically regulated by a number of effectors such as ATP, fructose 1,6-bisphosphate and gluconeogenic amino acids. This control prevents a futile loop between the enzymes of glycolysis and gluconeogenesis in tissues that can undertake both pathways. The M1 isoenzyme from skeletal muscle is not allosterically regulated and displays hyperbolic kinetics under physiological conditions.

It has recently been shown that the M1 and M2 isoenzymes are encoded by the same structural gene and that the differences in sequence arise by a tissue-specific mRNA-splicing event [3]. Moreover, the sequences of the two isoenzymes differ by only 21 amino acids clustered in two  $\alpha$ -helices which form the major intersubunit contact region across the 1,3-intersubunit interface [4]. It is therefore suspected that these two  $\alpha$ -helices, designated Ca1 and Ca2, are largely and perhaps solely involved in conferring the allosteric nature of the M1 isoenzyme because sequences throughout this region are identical in the M1 and M2 forms.

The yeast *Saccharomyces cerevisiae* has only one pyruvate kinase which is allosterically controlled and is reported to be kinetically most like the M2 isoenzyme. The gene has been cloned and sequenced [5] and is present on a 7 kbp *HindIII* fragment of DNA.

The yeast enzyme has been chosen for the present study because recent developments in the genetic manipulation of yeast have made it easy to delete wild-type genes and to overexpress proteins from high-copy-number vectors, thus facilitating purification and subsequent kinetic analysis (see [6] for a review).

The crystal structure of the cat M1 isoenzyme has been resolved to 2.6 Å [4] and the sequence of the yeast enzyme has been modelled into the cat muscle co-ordinates to give an approximation of its three-dimensional structure (T. H. L. Muirhead & H. Muirhead, Department of Biochemistry, University of Bristol, personal communication).

Three mutants have been made by oligonucleotide-directed mutagenesis in the 1,3-intersubunit contact area. Examination of the yeast structural model shows that serine-14 from subunit 1 lies directly opposite serine-414 from subunit 3. The change of this residue to one bearing a bulky or charged side chain is expected to force the 1,3-intersubunit interface apart, and thus yield a dimeric pyruvate kinase which is predicted to be catalytically active but no longer regulated by allosteric effectors. This residue has been changed to an arginine, a histidine and a proline. A further

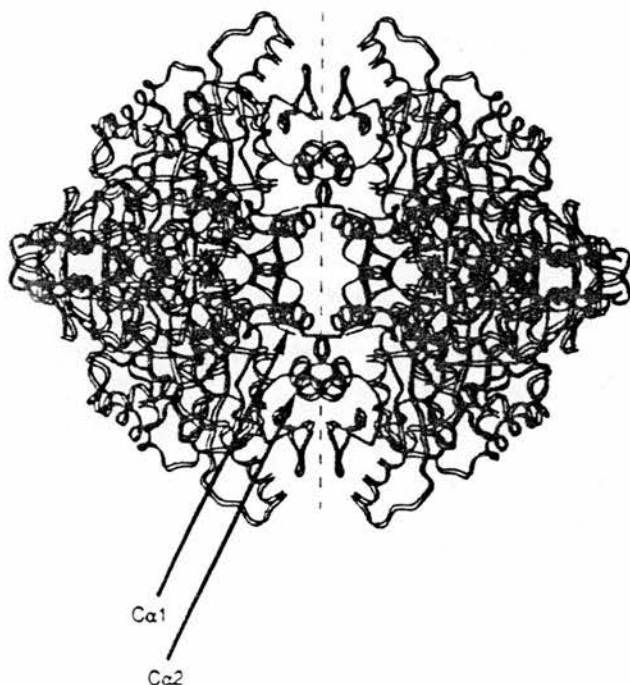


Fig. 1. A tetramer of pyruvate kinase

The broken line shows the 1,3-intersubunit interface. Ca1 and Ca2 are indicated.

series of mutations has been designed to look at the catalytic lysine-269 which is thought to stabilize the phospho intermediate as it is transferred from phosphoenolpyruvate to ADP. Lysine-269 has been changed to an arginine and a histidine to test the effect of moving the positive charge.

All of these mutations have been made at the DNA level and work is currently underway to overexpress the mutant proteins in yeast and to analyse their kinetic properties. A *pyk<sup>-</sup>* strain of yeast has been constructed in our laboratory by a simple gene disruption technique. Mutants which produce active protein can be easily identified by their ability to rescue the *pyk<sup>-</sup>* phenotype of the strain. Active mutants will be analysed by standard kinetic techniques to determine alterations in  $K_m$  and  $V_{max}$ , and in their susceptibility to allosteric regulators.

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